REMARKS/ARGUMENTS

Oath/Declaration

The Office Action indicates that the oath or declaration is defective because non-initialed and/or non-dated alterations have been made to the declaration filed on May 28, 2002 by inventor Tong Zhu. Applicants submit concurrently herewith a supplemental declaration in compliance with 37 C.F.R. § 1.67(a)(2) that has been executed by inventor Tong Zhu, indicating his citizenship as the United States of America and identifying the entire inventive entity.

The Objection to the Specification Should Be Withdrawn

The specification has been objected to for the incorporation of essential material therein by reference to a non-patent publication, a foreign application, or a foreign patent. The objection concerns material incorporated by reference on pages 28 (lines 27-28), 33 (line 7), 36 (lines 15-16), 39 (line 4), 55 (line 13), and 62 (lines 6-9). The Office Action indicates that "Applicant is required to amend the disclosure to include the material incorporated by reference" and that "[t]he amendment must be accompanied by an affidavit or declaration executed by the applicant, or a practitioner representing the applicant, stating that the amendatory material consists of the same material incorporated by reference in the referencing application." (Paper No. 06082004, p. 3)

Applicants respectfully disagree with this position of the Office Action. The Examiner is respectfully reminded that pursuant to M.P.E.P. § 608.01(p) "[n]onessential subject matter may be incorporated by reference to (1) patents or applications published by the United States or a foreign countries or regional patent offices . . . or (3) non-patent publications . . . ." In the Office Action, the Examiner has merely asserted that the material incorporated by reference is essential material and has failed to provide a single reason to support such an assertion. Accordingly, Applicants respectfully invite the Examiner in the next Office Action to set forth with particularity, for each of the occurrences cited in the Office Action, one or more reasons why the

material that has been incorporated by reference is essential. Otherwise, this objection to the specification should be withdrawn.

The Specification Has Been Amended to Insert SEQ ID NOS and to Delete "http://"

The Office Action indicates that the application fails to comply with the requirements of 37 C.F.R. §§ 1.821 through 1.825 concerning applications containing nucleotide and/or amino acid sequence disclosures. The Office Action indicates that the failure to comply with said requirements concerns the recitation of "MWLQP" on page 43, line 30 and "DYYT" on page 52, line 2 of the specification. The Office Action indicates that if these amino acids lie within one of the disclosed sequences of the Sequence Listing, amendment of the specification to indicate their location within a disclosed sequence would suffice.

Applicants have amended the specification as recommended by the Examiner and have also corrected an inadvertent typographical error. On page 43, Applicants have replaced the "MWLQP" on page 43, line 30 with --MWLKQP (SEQ ID NO:34)--. The replacement of "MWLQP" with --MWLKQP-- corrects an inadvertent typographical error and does not introduce new matter because the conserved motif MWLKQP is disclosed in the specification on page 45 at line 21 and in SEQ ID NO:34 of the Sequence Listing. Applicants have also inserted --(amino acid positions 1-4 of SEQ ID NO:32)-- immediately after "DYYT" on page 52 at line 2 of the specification.

In addition, Applicants have made further amendments to the specification on page to insert SEQ ID NOS. On page 45 of the specification, Applicants have inserted --(SEQ ID NO:22)-- immediately after "TGPNM" in line 7 and --(SEQ ID NO:23)-- immediately after "FATHY" in line 8. Applicants note that the original specification indicates on page 42 at line 30 that TGPNM corresponds to SEQ ID NO: 22 and further indicates on page 43 at line 1 that FATHY corresponds to SEQ ID NO: 23.

Applicants have further amended the specification to delete a hyperlink or other form of browser executable code in the specification. At page 16, line 21, Applicants have deleted "http://".

The amendments to the specification are purely formal in nature and do not introduce new subject matter.

In view of the amendments, it is submitted that the specification is in compliance with 37 C.F.R. §§ 1.821 through 1.825.

Status of the Claims

Claims 1, 3, 7, 8, 17, 18, 24, and 34 have been cancelled without prejudice or disclaimer. Claims 2, 4, 9, 16, 19-21, 25, 26, 28, 29, and 35-37 have been amended. New claims 38-43 have been added.

Claims 1, 3, 7, 17, and 18 have been cancelled because these claims are directed to non-elected subject matter. In response to claim rejections under 35 U.S.C. § 112, second paragraph, that are further discussed below, Applicants have cancelled claims 24 and 34 for the purpose of furthering prosecution and not to limit the scope of their claimed invention. Applicants note for the record that the subject matter of dependent claims 24 and 34 is encompassed by the claims from which these claims depend, claims 19 and 30, respectively. Furthermore, Applicants note for the record that the cancellation of claims 24 and 34 is neither intended as a disclaimer of the subject matter of these claims nor to limit the scope of any one of the claims from which these cancelled claims depend. Applicants expressly reserve the right to file one or more continuing applications to protect the subject matter of the cancelled claims.

Claims 2 and 26 have been amended to more distinctly point out Applicants' claimed invention. In particular, part (c) has been amended to replace "1-266" with --1-265-- to correct an inadvertent typographical error, parts (e) and (f) have been amended to recite that the "nucleotide sequence encodes a protein comprising mismatch-repair activity". Part (e) has been further amended to recite that "percent sequence identity is obtained using GAP version 10 with a GAP Weight of 50 and a Length Weight of 3." Part (g) has been amended to recite "a nucleotide sequence that hybridizes under stringent conditions to the complement of at least one nucleotide sequence selected from the group consisting of SEQ ID NOS:1 and 3, wherein said nucleotide sequence encodes a protein comprising mismatch-repair activity and said stringent

conditions comprise hybridization in a solution comprising 50% formamide, 1 M NaCl, and 1% SDS at 37°C and a wash in a solution comprising 0.1X SSC at 60°C". Part (h) has been amended to recite that the fragment or variant "has at least 85% sequence identity to at least one amino acid sequence selected from the group consisting of the amino acid sequences set forth in SEQ ID NOS: 2 and 4, and wherein percent sequence identity is obtained using GAP version 10 with a GAP Weight of 50 and a Length Weight of 3". Support for these amendments to claims 2 and 26 can be found throughout the specification particularly on page 7 at lines 4-6, page 8 at lines 14-30, page 9 at lines 3-5, page 10 at lines 13-26, page 13 at lines 9-22, page 16 at lines 22-29, and page 51 at lines 4-6, 14-16 and 26-30.

Claims 2 and 26 have been further amended to add new parts (i) and (j). Part (i) recites "a nucleotide sequence encoding an amino acid sequence having at least 85% sequence identity to at least one amino acid sequence selected from the group consisting of the amino acid sequences set forth in SEQ ID NOS: 2 and 4, wherein said nucleotide sequence encodes a protein comprising mismatch-repair activity and percent sequence identity is obtained using GAP version 10 with a GAP Weight of 50 and a Length Weight of 3" and part (j) recites "nucleotides 1-797 of SEQ ID NO: 1." Both parts (i) and (j) are fully supported by the specification. In particular, support can be found in the specification on page 8 at lines 14-30, page 9 at lines 1-14, page 16 at lines 22-29, and page 51 at lines 26-30.

Claim 19 has been amended to delete "the" immediately before "said recombination frequency". This amendment was made to remove a redundancy (i.e., "the said") from claim 19.

Claim 33 has been amended to point out more distinctly that "said nucleic acid molecule confers a dominant negative phenotype on said plant or at least one cell thereof". Support for this amendment to the claim can be found in original claim 26 and in the specification, particularly on page 22 at lines 15-30.

The following amendments to the claims, which are further discussed below, have been made in response to claims rejections under 35 U.S.C. § 112, second paragraph. These amendments are purely formal in nature are not intended to reduce the scope of the claims.

Furthermore, these amendments are fully supported by the specification and original claims, particularly original claims 2, 25, 28, 29, and 35.

Claim 4 has been amended to replace "nucleotide sequence" in claim line 2 with --nucleic acid molecule--.

Claim 9 has been amended to replace "nucleotide" in claim line 3 with --nucleic acid--.

Claim 16 has been amended to replace "nucleotide" in claim line 3 with --nucleic acid--.

Claim 19 has been amended to replace "nucleotide" in claim line 2 with --nucleic acid--.

Claim 20 has been amended to recite that the "nucleic acid molecule is operably linked to a promoter that drives expression in a plant cell."

Claim 21 has been amended to replace "nucleotide sequence" in claim line 2 with --nucleic acid molecule--.

Claim 25 has been amended to recite that "efficiency of chimeraplasty is increased in said plant or at least one cell thereof." Additional support for this amendment can be found, for example, in the specification on page 4 at lines 14-18, page 5 at lines 20-22, page 21 at lines 12-16, page 22 at lines 12-17, page 24 at lines 6-15, and page 26 at lines 10-13.

Claim 26 has been further amended as recommended by the Examiner to insert --nucleic acid molecule comprising a-- immediately before "nucleotide sequence" in claim line 2.

Claim 28 has been amended to clarify that "the efficiency of chimeraplasty is increased in said plant." Additional support for this amendment can be found, for example, in the specification on page 4 at lines 14-18, page 5 at lines 20-22, page 21 at lines 12-16, page 22 at lines 12-17, page 24 at lines 6-15, and page 26 at lines 10-13.

Claim 29 has been amended to point out more distinctly that "mismatch repair is altered in said plant." Additional support for this amendment can be found, for example, in the specification on page 4 at lines 13-14 and page 20 at lines 1-9.

Claim 35 has been amended to clarify that "efficiency of chimeraplasty is increased." Additional support for this amendment can be found, for example, in the specification on page 4 at lines 14-18, page 5 at lines 20-22, page 21 at lines 12-16, page 22 at lines 12-17, page 24 at lines 6-15, and page 26 at lines 10-13.

Claim 36 has been amended to replace "nucleotide" in claim line 2 with --nucleic acid--.

Claim 37 has been amended to replace "nucleotide sequence" in claim line 3 with --nucleic acid molecule--.

New claims 38-43 have been added. Support for the new claims can be found in original claims 2 and 9, and throughout the specification, particularly on page 8 at lines 14-30, page 9 at lines 1-14, page 16 at lines 22-29, page 20, page 21 at lines 1-6, page 29 at lines 27-29, page 32 at lines 3-30, and page 33.

No new matter has been added by way of the amendments to the claims or by the addition of the new claims.

Claims 2, 4-6, 9-16, 19-23, 25-33, and 35-43 are pending.

Reexamination and reconsideration of the application as amended are respectfully requested in view of the following remarks.

Claims 21, 24, 25, 28, 34 and 35 Are Free of the Prior Art

Applicants respectfully acknowledge that the Examiner has determined that "[c]laims 21, 24, 25, 28, 34, and 35 are free of the prior art, which neither teaches nor fairly suggests using

anitsense suppression, cosuppression or chimeraplasty of a plant MSH2 encoding nucleic acid to alter DNA repair processes." (Office Action mailed June 21, 2004, p. 18)

The Objection to Claim 29 Should Be Withdrawn

Claim 29 has been objected to as being of improper dependent form for failing to further limit the subject matter of a previous claim. The Office Action asserts that this claim does not further limit the method of claim 26 because said claim appears to be directed to an inherent property of the method of claim 26. The Office Action indicates that Applicants are required to cancel claim 29, amend the claim to place the claim in proper dependent form, or rewrite the claim in independent form.

While Applicants respectfully disagree with the view of the Office Action that claim 29 is an improper dependent claim because it does not further limit the subject matter of claim 26, Applicants—in the interest of furthering prosecution and not to limit the scope of their claimed invention—have amended claim 29 to point out more distinctly that "mismatch repair is altered in said plant."

In view of the amendment and remarks, it is submitted that the objection to claim 29 should be withdrawn.

The Rejection of the Claims under 35 U.S.C. § 112, Second Paragraph, Should Be Withdrawn

Claims 2, 4-6, 9-16, and 19-37 have been rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. Claims 24 and 34 have been cancelled. Claims 2, 4, 9, 16, 19-21, 25, 26, 28, 29, and 35-37 have been amended. New claims 38-43 have been added. This rejection is respectfully traversed and should not be applied to the newly submitted claims.

The Office Action indicates that part (g) of claim 2 is indefinite for the recitation of "stringent conditions" because the specification only provides general guidance and does not

teach the metes and bounds of the claimed nucleic acid. The Office Action also indicates that part (h) of claim 2 is indefinite for the recitation of "or variant of" because the specification does not teach the metes and bounds of the claimed nucleic acid.

Applicants have amended part (g) of claim 2 to recite "stringent conditions comprise hybridization in a solution comprising 50% formamide, 1 M NaCl, and 1% SDS at 37°C and a wash in a solution comprising 0.1X SSC at 60°C". While the specification sufficiently defines what is intended by a variant protein such that one of ordinary skill in the art would not find part (h) indefinite, Applicants have amended this part to recite that the variant amino acid sequence "has at least 85% sequence identity to at least one amino acid sequence selected from the group consisting of SEQ ID NOS: 2 and 4, and wherein percent sequence identity is obtained using GAP version 10 with a GAP Weight of 50 and a Length Weight of 3". Accordingly, amended claim 2 is not indefinite.

The Office Action indicates that claim 4 is indefinite because "nucleotide sequence" lacks proper antecedent basis in claim 2 directed to an isolated nucleic acid molecule. In contrast to the view of the Office Action, there is proper antecedent basis for "nucleotide sequence", which is recited multiple times in claim 2. In the interest of furthering prosecution and not to limit the scope of the claim, Applicants have amended claim 4 to replace "nucleotide sequence" in claim line 2 with --nucleic acid molecule--. Accordingly, amended claim 4 is not indefinite.

The Office Action indicates that claims 9, 16, and 19 are indefinite because "nucleotide molecule" lacks proper antecedent basis in claim 2 directed to an isolated nucleic acid molecule. While those of ordinary skill in the art would find that the recitation of "nucleic acid molecule" in claim 2 provides sufficient antecedent basis for "nucleotide molecule", Applicants have amended claims 9, 16, and 19 to replace "nucleotide molecule" with --nucleic acid molecule-- in the interest of furthering prosecution and not to limit the scope of the claim. Accordingly, amended claims 9, 16, and 19 are not indefinite.

The Office Action indicates that claim 20 is indefinite because "nucleotide construct" and "said nucleotide sequence" lack proper antecedent basis in claim 19. The Office Action indicates that "said nucleotide sequence" should read --nucleic acid molecule--. In contrast to the view of

the Office Action, there is proper antecedent basis for "nucleotide sequence", which is recited multiple times in claim 2, the claim from which claim 19 depends. In the interest of furthering prosecution and not to limit the scope of the claim, Applicants have amended claim 20 to recite that the "nucleic acid molecule is operably linked to a promoter that drives expression in a plant cell." Accordingly, amended claim 20 is not indefinite.

The Office Action indicates that claim 21 is indefinite because "said nucleotide sequence" lacks proper antecedent basis. As discussed above, there is proper antecedent basis for "nucleotide sequence", which is recited multiple times in claim 2, the independent claim from which claim 21 depends. In the interest of furthering prosecution and not to limit the scope of the claim, Applicants have amended claim 21 to replace "nucleotide sequence" in claim line 2 with --nucleic acid molecule--. Accordingly, amended claim 21 is not indefinite.

The Office Action indicates that claims 24 and 25 are indefinite because it is unclear if an additional method step is intended or if the introducing step at claim 19 is being further limited. The rejection of claim 24 is obviated by the cancellation of this claim. Applicants have amended claim 25 to clarify that "the efficiency of chimeraplasty is increased in said plant or at least one cell thereof." Accordingly, amended claim 25 is not indefinite.

The Office Action indicates that claim 26 is indefinite for the recitation of "nucleotide sequence" at line 2 because "nucleotide sequence" denotes information and not a composition of matter. The Office Action also indicates that part (g) of claim 26 is indefinite for the recitation of "stringent conditions" because the specification only provides general guidance and does not teach the metes and bounds of the claimed nucleic acid. The Office Action also indicates that part (h) of claim 26 is indefinite for the recitation of "or variant of" because the specification does not teach the metes and bounds of the claimed nucleic acid.

Applicants have amended claim 26 at line 2 to recite --nucleic acid molecule comprising a nucleotide sequence-- as recommended in this Office Action. As they have done for claim 2, Applicants have amended part (g) of claim 26 to recite "stringent conditions comprise hybridization in a solution comprising 50% formamide, 1 M NaCl, and 1% SDS at 37°C and a wash in a solution comprising 0.1X SSC at 60°C". While the specification sufficiently defines

what is intended by a variant protein such that one of ordinary skill in the art would not find part (h) indefinite, Applicants have amended this part to recite that the variant amino acid sequence "has at least 85% sequence identity to at least one amino acid sequence selected from the group consisting of SEQ ID NOS: 2 and 4, and wherein percent sequence identity is obtained using GAP version 10 with a GAP Weight of 50 and a Length Weight of 3". Accordingly, amended claim 26 is not indefinite.

The Office Action indicates that claims 28, 34, and 35 are indefinite because it is unclear if an additional method step is intended or if the introducing at claim 26 is being further limited. The rejection of claim 34 is obviated by the cancellation of this claim. Applicants have amended claims 28 and 35 to clarify that "efficiency of chimeraplasty is increased in said plant." Accordingly, amended claims 28 and 35 are not indefinite.

The Office Action indicates that claim 36 is indefinite because "nucleotide molecule" lacks proper antecedent basis in claim 2 directed to an isolated nucleic acid molecule. While those of ordinary skill in the art would find that "nucleic acid molecule" in claim 2 provides sufficient antecedent basis for "nucleotide molecule" in claim 2, Applicants have amended claim 36 to replace "nucleotide molecule" with --nucleic acid molecule-- in the interest of furthering prosecution and not to limit the scope of the claim. Accordingly, amended claim 36 is not indefinite.

The Office Action indicates that claim 37 is indefinite because "nucleotide sequence" lacks proper antecedent basis in claim 36. As discussed above, there is proper antecedent basis for "nucleotide sequence", which is recited multiple times in claim 2, the independent claim from which claim 36 depends. In the interest of furthering prosecution and not to limit the scope of the claim, Applicants have amended claim 37 to replace "nucleotide sequence" in claim line 3 with --nucleic acid molecule--. Accordingly, amended claim 37 is not indefinite.

In view of the amendments and remarks, it is submitted that the rejection of the claims under 35 U.S.C. § 112, second paragraph, should be withdrawn and not be applied to the newly submitted claims.

The Rejections of the Claims Under 35 U.S.C. § 112, First Paragraph, Should Be Withdrawn

Claims 2, 4-6, 9-16, and 19-37 have been rejected under 35 U.S.C. § 112, first paragraph. Claims 24 and 34 have been canceled. Claims 2, 4, 9, 16, 19-21, 25, 26, 28, 29, and 35-37 have been amended. New claims 38-43 have been added. This rejection is respectfully traversed and should not be applied to the newly submitted claims.

Written Description

Claims 2, 4-6, 9-16, and 19-37 have been rejected under 35 U.S.C. § 112, first paragraph, for lack of adequate written description. The Office Action indicates that Applicants claim an isolated nucleic acid molecule comprising at least 85% sequence identity to SEQ ID NO: 1 or 3, at least 50 contiguous nucleotides of SEQ ID NO: 1 or 3, that hybridizes under stringent conditions to a nucleotide having the nucleotide sequence of SEQ ID NO: 1 or 3, or encoding a fragment or variant of the amino acid sequence of SEQ ID NO: 2 or 4 that confers a dominant-negative phenotype in a host cell. The Office Action further indicates that that Applicants claim a method of altering recombination frequency in a plant comprising introducing such a nucleic acid molecule and plants transformed to comprise such a nucleic acid molecule.

The Office Action indicates that Applicants describe SEQ ID NO: 1 encoding SEQ ID NO: 2 and SEQ ID NO: 3 encoding SEQ ID NO: 4 both nucleic acid molecules encoding tobacco MSH2 repair protein involved in mismatch repair. The Office Action further indicates that Applicants describe a 265 amino acid N-terminal fragment of said MSH2 protein that produces a mutator phenotype when expressed in *E. coli*. The Office Action asserts that Applicants do not describe the genus of molecules comprising a nucleotide sequence with at least 85% sequence identity to SEQ ID NO: 1 or 3, comprising at least 50 contiguous nucleotides of SEQ ID NO: 1 or 3, that hybridizes under stringent conditions to a nucleotide having the nucleotide sequence of SEQ ID NO: 1 or 3, or encoding a fragment or variant of the amino acid sequence of SEQ ID NO: 2 or 4 that confers a dominant-negative phenotype in a host cell. The Office Action concludes that it is unclear from the instant specification that Applicants were in possession of the invention as broadly claimed. In support of this position, the Office Action

cites *Regents of the University of California v. Eli Lilly and Co.*, 43 U.S.P.Q. 2d 1398 (Fed. Cir. 1997).

As described above, parts (e) and (f) of claims 2 and 26 have been amended to recite that the nucleotide sequence encodes a protein comprising mismatch-repair activity. Part (e) has been further amended to recite that percent sequence identity is obtained using GAP version 10 with a GAP Weight of 50 and a Length Weight of 3. Part (g) of these claims has been amended to recite that stringent conditions comprise hybridization in a solution comprising 50% formamide, 1 M NaCl, and 1% SDS at 37°C and a wash in a solution comprising 0.1X SSC at 60°C and that the nucleotide sequence encodes a protein comprising mismatch-repair activity. Part (h) of these claims has been amended to the recite that nucleotide sequence encodes a fragment or variant the has at least 85% sequence identity to at least one amino acid sequence selected from the group consisting of SEQ ID NOS: 2 and 4 and that percent sequence identity is obtained using GAP version 10 with a GAP Weight of 50 and a Length Weight of 3.

Amended claims 2 and 26, as *Lilly* requires, recite the functional and structural features of the claimed isolated nucleotide sequences. Amended claims 2 and 26 recite that the fragment and variant nucleotide sequences encode proteins comprising mismatch-repair activity or are the complements of such nucleotide sequences. The specification provides adequate description of the subject matter of the amended claims and its dependent claims so as to reasonably convey to one skilled in the relevant art that Applicants had possession of the invention as claimed. In particular, the specification discloses on pages 7-11 that the invention encompasses fragments and variants of the disclosed nucleotide sequence, wherein such fragments and variants encode proteins comprising mismatch-repair activity. Accordingly, the subject matter of amended claims 2 and 26 and their respective dependent claims is adequately described in the instant specification so as to reasonably convey to one of ordinary skill in the relevant art that, at the time of the invention, Applicants had possession of the claimed invention. The written description requirement of 35 U.S.C. §112, first paragraph, has been satisfied.

In summary, in view of the amendments and above remarks, claims 2, 4-6, 9-19, and 19-37 satisfy the written description requirement of 35 U.S.C. §112, first paragraph, and the

Examiner is respectfully requested to withdraw the rejection and not apply it to the newly submitted claims.

Enablement

Claims 2, 4-6, 9-16, and 19-37[1] have been rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. The Office Action asserts that given Applicants' limited guidance it would have required undue trial and error experimentation by one of ordinary skill in the art at the time of Applicants' invention to practice the invention as broadly claimed.

The Office Action indicates that the specification is enabled "for an isolated nucleic acid molecule encoding the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3, or encoding a fragment encoding residues 1-266 thereof of, and methods of using said isolated nucleic acid molecule." (Paper No. 06082004, p. 9)

Applicants note that, while SEQ ID NO: 2 of the instant Sequence Listing is an amino acid sequence, SEQ ID NO: 3 is a nucleotide sequence. Applicants assume that the Examiner intended to indicate the specification is enabled for an isolated nucleic acid molecule encoding the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4. If this is not the case, Applicants respectfully request a further opportunity to respond to this rejection of the claims.

The Office Action asserts that the specification does not provide reasonable enablement for an isolated nucleic acid molecule comprising the nucleotide sequence having at least 85% sequence identity to SEQ ID NO: 1 or 3, comprising at least 50 contiguous nucleotides of SEQ ID NO: 1 or 3, that hybridizes under stringent conditions to a nucleotide having the nucleotide sequence of SEQ ID NO: 1 or 3, and encoding a fragment or variant of the amino acid sequence of SEQ ID NO: 2 or 4 that confers a dominant-negative phenotype in a host cell. In support of this position, the Office Action cites *In re Wands* 8 U.S.P.Q. 2d 1400 (Fed. Cir. 1988).

[1] The Office Action indicates that "[c]laims 2, 4-6, 9-16 and ***19-3*** are rejected under 35 U.S.C. § 112, first paragraph . . . ." (Paper No. 06082004, p. 9) (emphasis added) Applicants assume that the Examiner intended for claims 19-37 to be part of the enablement rejection. If this is not the case, Applicants respectfully request that the Examiner set forth in the next Office Action, the claims that are part of the enablement rejection.

The Office Action asserts that art of making and using nucleic acid molecules that encode mismatch repair proteins, particularly MSH2 proteins is complex, that the MSH2 protein is part of a protein complex, and that the Bowers *et al.* reference ((2000) *J. Mol. Biol.* 302:327-338) teaches that MSH2 binds ATP and forms a complex with several other proteins in a particular manner. The Office Action concludes that Applicants have provided limited guidance on how to make and use nucleic acid molecules that encode the fragments and variants of the exemplified tobacco MSH2 proteins as broadly claimed. Applicants respectfully disagree with this position of the Office Action as is discussed in detail below.

The Office Action further asserts that the Pang *et al.* reference ((1997) *Mol. Cell Biol.* 17:4465-4473) teaches that a yeast Pms1p truncation mutation did not have a dominant-negative effect and that dominant-negative effect phenotype in the *E. coli.* MutL protein appears to involve a conserved N-terminal portion of Pms1p and apparently a high level of expression. However, Applicants' invention is not directed to a Pms1 protein or an Msh1 protein but is instead directed to an MSH2 protein. The Office Action then extends the teachings of Pang *et al.*—without any providing any additional support—to encompass all mismatch repair proteins in reaching the conclusion that "the art teaches that it is unpredictable whether a truncated to modified mismatch repair protein will actually affect mismatch repair function in a cell without empiric evidence." (Paper No. 06082004, p. 12) Applicants respectfully remind the Examiner that the teachings of the Pang *et al.* reference are directed to the Pms1 and Msh1 proteins, not the MSH2 proteins of the instant invention.

The Office Action concludes that it cannot be predicted by one of ordinary skill in the art that nucleic acids that are at least 85% identical will encode a protein with the same activity as either SEQ ID NO: 1 or 3 and cites the teachings of the Bowie *et al.* ((1990) *Science* 247:1306-1310), Lazar *et al.* ((1988) *Mol. Cell. Biol.* 8:1247-1252) and Broun *et al.* ((1998) *Science* 282:1315-1317) references in support of this view. The Office Action concludes that the Lazar *et al.* and Broun *et al.* references demonstrated that one or a few amino acid substitutions could dramatically affect the biological activity and structure-function characteristics of a protein in unpredictable ways.

The Office Action, however, fails to indicate that the Bowie *et al.* reference teaches that guidance on allowed amino acid substitutions can be obtained through alignments with other members of a protein family. While those of the ordinary skill in the art do not need guidance to conduct such sequence alignments of MSH2 protein family members, the specification provides additional guidance on pages 14-19. In addition, the specification provides the accession numbers for six MSH2 proteins that were known in the art at the time of the invention. Thus, one of ordinary skill in the art would, in view of the instant specification, would be able to easily prepare an amino acid sequence alignment of known MSH2 proteins and identify amino acids positions in the exemplified MSH2 protein of the invention that would likely allow for an amino acid substitution without loss of function. Additionally, those positions that would likely not allow for an amino acid substitution without loss of function or a reduction in mismatch repair activity can be similarly identified.

In addition, the Lazar *et al.* reference states: "[w]hen aspartic acid 47 was mutated to alanine or asparagine, biological activity was retained..." (page 1247, Abstract). Thus, the Lazar *et al.* reference demonstrates that not all substitutions, including non-conservative ones as noted above, impact the biological activity of a protein. Furthermore, the Office Action fails to mention that the teachings of the Lazar *et al.* reference concern transforming growth factor α (TGF-α), a very small polypeptide of only 50 amino acids that is a peptide hormone. In contrast, the two exemplified MSH2 proteins of the present invention (SEQ ID NOS: 2 and 4), which are not known to be peptide hormones, are each 939 amino acids in length. Thus, one of ordinary skill in the art would likely not view the teachings of the Lazar *et al.* reference as being generally applicable to any protein having any number of amino acids.

The Office Action also fails to indicate that the Broun *et al.* reference teaches that the four amino acid substitutions in an oleate 12-desaturase that converted the enzyme from a strict desaturase to a mixed function enzyme having both desaturase and hydroxylase activities were among the seven amino acid positions that were strictly conserved across six plant oleate 12-desaturases, including desaturases from both monocot and dicot plants. Furthermore, Broun *et al.* teach that these four substituted amino acid positions are adjacent to histidine residues that have been identified as essential for catalysis. In view of the general knowledge in the art and in

further view of the teachings of the Bowie *et al.* reference discussed above, one of ordinary skill in the art would expect that amino acid substitutions of Broun *et al.*, which were at highly conserved positions within a catalytic site of an enzyme, would not be allowed amino acid substitutions for the production of a variant enzyme that retained the catalytic function of the wild-type enzyme.

As discussed above, parts (e), (f) and (g) of claims 2 and 26 have been amended to recite that the nucleotide sequence encodes a protein comprising mismatch repair activity. Part (e) has been further amended to recite that percent sequence identity is obtained using GAP version 10 with a GAP Weight of 50 and a Length Weight of 3. Part (g) has been further amended to recite that the stringent conditions comprise hybridization in a solution comprising 50% formamide, 1 M NaCl, and 1% SDS at 37°C and a wash in a solution comprising 0.1X SSC at 60°C. Part (h) has been amended to recite that the fragment or said variant confers a dominant-negative phenotype in a host cell and has at least 85% sequence identity to at least one amino acid sequence selected from the group consisting of SEQ ID NOS: 2 and 4 and that percent sequence identity is obtained using GAP version 10 with a GAP Weight of 50 and a Length Weight of 3.

In contrast to the conclusions of the Office Action, the specification provides sufficient guidance to make and identify the isolated nucleotide molecules encompassed by the claims. In particular, Applicants have provided the nucleotide sequences of SEQ ID NO: 1 and SEQ ID NO: 3 and the amino acid sequences of SEQ ID NO: 2 and SEQ ID NO: 4. The claimed nucleotide sequences vary from this sequence by structural parameters (*i.e.*, at least 85% identity to SEQ ID NO: 1 and/or 3, or at least at least 50 contiguous bases of SEQ ID NO: 1 and/or 3, hybridizes to SEQ ID NO: 1 and/or 3 under defined stringent conditions, or encodes a protein having at least 85% identity to SEQ ID NO: 2 and/or 4) that can be determined by those of ordinary skill in the art. While methods for sequence alignments, sequence comparisons, determining percent sequence identity, and hybridization are within the knowledge of one of ordinary skill in the art, additional guidance for is set forth in the specification on pages 12-19.

Moreover, the nucleotide sequences of the invention encode proteins comprising mismatch repair activity or that are capable of conferring a dominant-negative phenotype in a

host cell. Such nucleotide sequences include those that are fragments and variants of SEQ ID NOS: 1 and 3, and that encode proteins comprising mismatch repair activity. Such nucleotide sequences further include those that encode fragments and variants of SEQ ID NO: 2 and 4 that are capable of conferring a dominant-negative activity in a host cell. Methods for assaying whether the nucleotide sequences encode proteins comprising mismatch repair activity or that are capably of conferring such a dominant-negative phenotype are known in the art and are also provided in the instant specification on page 10 at lines 17-26 and in Example 5 (pp. 51-52) . Accordingly, based on the guidance in the specification, one of ordinary skill in the art would be able to make and used Applicants' claimed invention.

The Federal Circuit has repeatedly stated that enablement is not precluded by the necessity for some experimentation, so long as the experimentation needed to practice the invention is not undue. *In re Wands* 8 U.S.P.Q. 2d 1400 (Fed. Cir. 1988). Furthermore, a considerable amount of experimentation is permissible, if it is merely routine, or if the specification provides a reasonable amount of guidance in which the experimentation should proceed. *Id.*

Applicants stress that when evaluating the quantity of experimentation required, the court looks to the amount of experimentation required to practice a single embodiment of the invention, rather than the amount required to practice every embodiment of the invention. For example, in *Wands*, the claims at issue were drawn to immunoassay methods using any monoclonal antibody having a binding affinity for HbsAg of at least 10-9 M. The USPTO had taken the position that the claim was not enabled as it would take undue experimentation to make the monoclonal antibodies required for the assay. The Federal Circuit reversed, and held that the claims were enabled, as the amount of experimentation required to isolate monoclonal antibodies and screen for those having the correct affinity was not undue. *Id.* Clearly, the Federal Circuit did not contemplate that every antibody useful in the methods of the claim must be identified. Rather, the court considered the amount of experimentation required to identify one or a few monoclonal antibodies having the required affinity.

In the instant case, the quantity of experimentation required to practice the invention amounts to two steps, identifying a nucleotide sequence that comprises at least 85% sequence identity to the nucleotide sequence of SEQ ID NO: 1 and/or 3, has at least 50 contiguous bases of the nucleotide sequence of SEQ ID NO: 1 and/or 3, hybridizes to SEQ ID NO: 1 and/or 3 under defined stringent conditions, or encodes a protein having at least 85% identity to SEQ ID NO: 2 and/or 4 and then assaying the protein encoded thereby for functional activity. Thus, ample guidance is provided to allow one of skill in the art to identify additional nucleotide sequences encompassed by the claims 2 and 26 and their respective dependent claims. Consequently, contrary to the conclusions of the Office Action, the quantity of experimentation necessary and the amount of guidance presented in the specification is sufficient to enable Applicants' claimed invention. Accordingly, Applicants submit that claims 2, 4-6, 9-16, and 19-37 and the newly submitted claims are enabled under 35 U.S.C. §112, first paragraph.

The Office Action indicates that claims 21, 24, and 26-37, which are directed to methods using antisense sequence or cosuppression, are only deemed enabled to the extent that these claims are directed to a method in tobacco. The Office Action asserts that antisense suppression and cosuppression are unpredictable in a heterologous plant because of the requirement of complementation of the expressed message in the plant cell to suppress the expression of a protein encoding nucleic acid and that Applicants have provided no working examples of suppression or cosuppression of a MSH2 protein in a plant and only general guidance. The Office Action cites the Colliver *et al.* reference ((1997) Plant Mol. Biol. 35:509-522) as teaching expression of a bean chalchone synthase gene in *Lotus corniculatus* did not predictably suppress expression of the heterologous gene and in some cases increased expression of the heterologous gene. The Office Action additionally cites the Elomaa *et al.* reference ((1996) *Mol. Breeding* 2:41-51) as teaching that "expression of an antisense construct of a chalchone synthase gene gchs1 or gchs2, which are only 73% identical to each other, do not cross suppress expression of the endogenous genes, in fact overexpression of the antisense gchs1 gene had not suppressive effect . . . ." (Paper No. 06082004, p. 14)

Applicants are unable to comment on the teachings of the Elomaa *et al.* reference at this time because they were not able to review the reference in its entirety. The copy of the Elomaa

et al. reference that was forwarded to Applicants included pages 41-43 and 48-50, but did not include pages 44-47. Applicants kindly request that the Examiner forward to them with the next Office Action a complete copy of this reference or at least copies of the missing pages, pages 44-47. Furthermore, Applicants kindly request that they be afforded another opportunity to address fully the comments made in the present Office Action regarding the Elomaa *et al.* reference, after receiving having had an opportunity to review the reference in its entirety.

In contrast to the view of the Office Action, Applicants provide not only the exemplified nucleotide sequences set forth in claims 2 and 26 but also sufficient guidance for one of ordinary skill in the art to make and use the invention as claimed in any plant including methods that involve antisense suppression or cosuppression. While antisense suppression and cosuppression methods are known to those of ordinary skill in the art, the specification provides additional guidance on pages 34 and 35, including the teachings of U.S. Patent Nos. 5,283,184 and 5,034,323, which have been incorporated by reference into the specification.

The Office Action indicates that claims 25 and 28, which are directed to methods using chimeraplasty, lack adequate enablement. The Office Action asserts that the Applicant only provides generalized guidance on how to make and use the nucleotide constructs to practice this type of invention. The Office Action further asserts that the art teaches that using chimeraplasty to modify a coding sequence is not predictable and cites the teachings of Anderson *et al.* ((2000) *J. Mol. Med.* 80:770-781) and Liu *et al.* ((2003) *Nat. Rev. Genet.* 4:679-689) in support of this position.

Notwithstanding the Examiner's assertions, chimeraplasty has been used successfully to modify specific sites in plant genes in both monocots and dicots. As further evidence, Applicants provide herewith Beetham *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:8874-8778 (Appendix A) Zhu *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:8768–8773 (Appendix B), and Kochevenko *et al.* (2003) *Plant Physiology* 132:174-184 (Appendix C). The results provided in the Beetham *et al.*, Zhu *et al.*, and Kochevenko *et al.* references are summarized in Table 1 and clearly establish that only routine experimentation is required to practice the claimed invention.

Table 1.

Reference	Plant system	Target Site Modified	Pages of Reference
Beetham *et al.*	dicot	Pro196 of ALS gene	p. 8777, column 2, paragraph 2
Beetham *et al.*	dicot	Codon 6 of GFP transgene integrated into genome	p. 8778, column 1; p8875, column 1,lines 4-5
Zhu *et al.*	monocot	Ser621 of AHAS	Table 2, page 8871
Zhu *et al.*	monocot	Pro165 of AHAS	Table 2, page 8871
Kochevenko *et al*	dicot	Try573 of ALS gene	Table 1, p. 179
Kochevenko *et al*	dicot	Pro196 of ALS gene	Table 1, p. 179

As illustrated in Table 1, the Beetham *et al.*, Zhu *et al.*, and Kochevenko *et al.* references demonstrate the successful targeting of various positions in a target gene using chimeric oligonucleotides in plants. Thus, the data summarized in table 1 provides evidence that the use of chimeraplasty is successful in plants, and accordingly, contrary to the assertion by the Examiner, the use of chimeraplasty to modify a coding sequence is not unpredictable.

In the interest of furthering prosecution, Applicants have amended claim 25 and 28 to clarify that their claimed methods can be used to increase efficiency of chimeraplasty.

In view of the amendments and above remarks, it is apparent that those of skill in the art would be able to practice the present claims without undue experimentation. Accordingly, the enablement rejection of claims 2, 4-6, 9-16, and 19-37 should be withdrawn and not applied to the newly submitted claims.

The Rejection of the Claims Under 35 U.S.C. § 102(b) Should Be Withdrawn

Claim 2 has been rejected under 35 U.S.C. § 102(b) as being anticipated by the Culligan and Hays reference ((2000) *Plant Cell* 12:991-1002). Claim 2 has been amended. New claims 38-43 have been added. This rejection is respectfully traversed and should not be applied to the newly submitted claims.

The Office Action indicates that the Culligan and Hays reference discloses isolated nucleic acids encoding an *Arabidopsis thaliana* and a *Zea mays* MSH2 protein that would bind under stringent conditions to a nucleic acid having the sequence of SEQ ID NOS: 1 or 3 and that the nucleic acids disclosed by the Culligan and Hays reference would also be considered to encode variants of the amino acid sequence of SEQ ID NOS: 2 or 4. The Office Action concludes that the Culligan and Hays reference has previously disclosed the claim imitations.

In contrast to the view of the Office Action, the cited Culligan and Hays reference does not disclose nucleic acids encoding an *Arabidopsis thaliana* and *Zea mays* MSH2 proteins, but instead discloses the isolation of nucleic acids encoding *Arabidopsis thaliana* MSH3 (Accession No. AL022197), MSH6 (Accession No. AF001308), and MSH7 (Accession No. AF193018) proteins (p. 994, left column) and heterodimers with AtMSH2 (p. 991, Abstract). Furthermore, the nucleic acids disclosed by this reference would not hybridize under stringent conditions to a nucleic acid molecule having the sequence of SEQ ID NOS: 1 or 3 as is supported by percent sequence identities. For example, when SEQ ID NO: 1 of the present invention is aligned using GAP Version 10 with the nucleotide sequences of each of AL022197, AF001308, and AF193018, the calculated percent nucleotide sequence identities are only 44%, 41%, and 42%, respectively. When SEQ ID NO: 3 is similarly aligned with the nucleotide sequences of each of AL022197, AF001308, and AF193018, the calculated percent nucleotide sequence identities are only 43%, 41%, and 43%, respectively. Finally, the Culligan and Hays reference does not disclose nucleic acids that encode variants of SEQ ID NO: 2 and 4. When either SEQ ID NO: 2 or 4 is aligned using GAP Version 10 with the amino acid sequences of each of AL022197, AF001308, and AF193018, the calculated percent amino acid sequence identities are only 29%, 27%, and 32%, respectively.

Although the Culligan and Hays reference fails to disclose nucleic acids that are capable of hybridizing to SEQ ID NO: 1 or 3 under stringent conditions or that encode variants of the amino acid sequence of SEQ ID NOS: 2 or 4, Applicants have amended claim 2, part (g) to clarify that stringent conditions comprise hybridization in a solution comprising 50% formamide, 1 M NaCl, and 1% SDS at 37°C and a wash in a solution comprising 0.1X SSC at 60°C. Accordingly, amended claim 2 is not anticipated by the Culligan and Hays reference.

In view of the amendments and remarks, it is submitted that the rejection of claim 2 under 35 U.S.C. § 102(b) should be withdrawn and not be applied to the newly submitted claims.

The Rejection of the Claims Under 35 U.S.C. § 103(a) Should Be Withdrawn

Claims 4-6, 9-16, 19, 20, 22, 23, 26-27, 29-33, 36, and 37 have been rejected under 35 U.S.C. § 103(a) as being unpatentable over Chao *et al.* (U.S. Patent App. Pub. No. 2003/0143586 A1, filed October 11, 2002; claiming the benefit of U.S. Provisional Pat. App. Serial No. 60/328,750, filed October 12, 2001). Claims 4, 9, 16, 19, 20, 26, 29, 36, and 37 have been amended. New claims 38-43 have been added. This rejection is respectfully traversed and should not be applied to the newly submitted claims.

Applicants note that the Office Action indicates on page 17 at line 2 that the Chao *et al.* reference is "US-PGPUB 2003/0143286." However, this citation corresponds to a patent publication of Stevenson *et al.* entitled "Canine Health Diet." The Notices of References Cited (Form PTO-982) correctly indicated that the U.S. Patent App. Pub. No. 2003/0143586 A1. Applicants assume that the Examiner made an inadvertent typographical error on page 17 of the Office Action. If Applicants assumption is incorrect, Applicant respectfully request a further opportunity to respond to the rejection of the claims under 35 U.S.C. § 103(a) in response to the next Office Action.

The Office Action indicates that the Chao *et al.* reference teaches a method of making a plant transformed with an isolated nucleic acid encoding an AtMSH2 protein that operates as a dominant negative allele in the plant, that this method can be used to make transgenic *Brassica* sp. or monocot plants, and that such an dominant negative allele can comprise a truncation

mutation of a mismatch repair protein. The Office Action asserts that the AtMSH2 encoding nucleic acid (SEQ ID NO: 46) of Chao *et al.* would have been considered capable of hybridizing under stringent conditions with SEQ ID NOS: 1 or 3 of the instant application. The Office Action further indicates that the Chao *et al.* reference fails to teach plants transformed with an MSH2 dominant negative allele or constructs to make such plants. The Office Action concludes that it would have been prima facie obvious to one of ordinary skill in the art at the time of the invention to use the teachings of the Chao *et al.* reference to make an expression cassette comprising the AtMSH2 encoding nucleic acid or truncation thereof, operably linked to a plant operative promoter and transform a plant wherein said plant has increased recombination frequency and an altered DNA repair process. The Office Action additionally asserts that the plants taught by Chao *et al.* would have been viewed by one of skill in the art to be functional equivalents and would not have lead to a teaching of unexpected results as to the particular plant transformed.

In contrast to the view of the Office Action, the Chao *et al.* reference does not disclose nucleic acids that are capable of hybridizing under stringent conditions to SEQ ID NOS: 1 or 3 of the instant application. When SEQ ID NO: 46 of Chao *et al.* is aligned using GAP Version 10 with each of SEQ ID NOS: 1 and 3 of the present invention, the calculated percent identities are only 42% and 41%, respectively. Thus, the Chao *et al.* reference does not disclose the nucleic acids of Applicants' claimed invention.

Applicants have amended part (g) of claims 2 and 26 to clarify that stringent conditions comprise hybridization in a solution comprising 50% formamide, 1 M NaCl, and 1% SDS at 37°C and a wash in a solution comprising 0.1X SSC at 60°C. The Office Action does not indicate that the Chao *et al.* reference teaches nor renders obvious the nucleotide sequences of SEQ ID NO: 1 or 3, nucleotide sequences encoding SEQ ID NO: 2 or 4, or nucleotide sequence capable of hybridizing to SEQ ID NO: 1 or 3 under conditions comprising hybridization in a solution comprising 50% formamide, 1 M NaCl, and 1% SDS at 37°C and a wash in a solution comprising 0.1X SSC at 60°C. Thus, Applicants' claimed invention is patentable over Chao *et al.*

In view of the amendments and remarks, it is submitted that the rejection of claims 4-6, 9-16, 19, 20, 22, 23, 26-27, 29-33, 36, and 37 under 35 U.S.C. § 103(a) should be withdrawn and not be applied to the newly submitted claims.

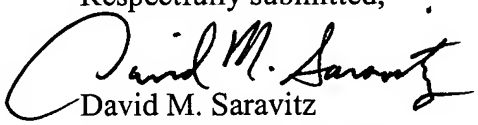
CONCLUSIONS

In view of the above amendments and remarks, Applicants submit that the rejections of the claims under 35 U.S.C. §§ 102, 103, and 112 are overcome. Applicants respectfully submit that this application is now in condition for allowance. Early notice to this effect is solicited. In any event, the Examiner is respectfully requested to enter the above amendments for the purpose of furthering prosecution.

If in the opinion of the Examiner a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned.

It is not believed that extensions of time or fees for net addition of claims are required, beyond those that may otherwise be provided for in documents accompanying this paper. However, in the event that additional extensions of time are necessary to allow consideration of this paper, such extensions are hereby petitioned under 37 CFR § 1.136(a), and any fee required therefore (including fees for net addition of claims) is hereby authorized to be charged to Deposit Account No. 16-0605.

Respectfully submitted,



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Karyn Grimm

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Plant Biology

A tool for functional plant genomics: Chimeric RNA/DNA oligonucleotides cause *in vivo* gene-specific mutations

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Contributed by Charles J. Arntzen, May 5, 1999

ABSTRACT **Self-complementary chimeric oligonucleotides (COs) composed of DNA and modified RNA residues were evaluated as a means to (*i*) create stable, site-specific base substitutions in a nuclear gene and (*ii*) introduce a frameshift in a nuclear transgene in plant cells. To demonstrate the creation of allele-specific mutations in a member of a gene family, COs were designed to target the codon for Pro-196 of SuRA, a tobacco acetolactate synthase (ALS) gene. An amino acid substitution at Pro-196 of ALS confers a herbicide-resistance phenotype that can be used as a selectable marker in plant cells. COs were designed to contain a 25-nt homology domain comprised of a five-deoxyribonucleotide region (harboring a single base mismatch to the native ALS sequence) flanked by regions each composed of 10 ribonucleotides. After recovery of herbicide-resistant tobacco cells on selective medium, DNA sequence analyses identified base conversions in the ALS gene at the codon for Pro-196. To demonstrate a site-specific insertion of a single base into a targeted gene, COs were used to restore expression of an inactive green fluorescent protein transgene that had been designed to contain a single base deletion. Recovery of fluorescent cells confirmed the deletion correction. Our results demonstrate the application of a technology to modify individual genetic loci by catalyzing either a base substitution or a base addition to specific nuclear genes; this approach should have great utility in the area of plant functional genomics.**

Genomics is currently a central component of plant biology research. The gene sequence of *Arabidopsis thaliana* will soon be available, and many other species are under study. Identification of new genes is occurring at a much faster pace than is the determination of their function. One missing technology in plant biology is the ability to selectively and reliably create site-specific "gene knockouts" or homologous recombination of genes of interest or of unknown function. The phenomena of gene silencing (e.g., antisense and cosuppression) provides a method for understanding gene function through the creation of transgenic crops expressing a gene sequence that silences the endogenous gene. However, this approach is not suitable for functional genomic studies of individual members of multigene families or genes with similar sequence, and is sometimes problematic in other technical aspects (1).

A technology currently being explored in prokaryotic and eukaryotic systems uses self-complementary chimeric oligonucleotides (COs) comprised of DNA and 2′-*O*-methyl RNA to target and mutate genes *in vivo*. These COs are designed to have one or more bases that do not pair with the endogenous gene sequence. This approach was successfully used to modify endogenous genes of mammalian cells (2–5) in a site-specific and genetically inheritable manner. Recently, Alexeev and Yoon (5) have demonstrated that permanent *in vivo* conversions result in phenotypic changes in mouse melanocytes. It has been hypothesized that the mechanism by which COs induce mutation is via action of DNA repair enzymes that recognize mismatches between the targeted gene and the CO, which was designed to create a mismatch (6).

Sulfonylurea herbicides retard plant growth by inhibiting branched-chain amino acid biosynthesis by blocking acetolactate synthase (ALS) (7). ALS is encoded by a diallelic gene family in *Nicotiana tabacum* (an allotetraploid species). The herbicides are no longer toxic to plants that contain at least one mutated gene encoding an altered form of ALS. A mutation that causes an amino acid substitution at Pro-196 confers resistance to the herbicide chlorsulfuron (Glean, DuPont) but the enzyme remains sensitive to another herbicide, imazaquin (Scepter, American Cyanamid) (8–10).

In this manuscript, we aimed to determine whether the introduction of COs is sufficient to cause targeted mutations in a nuclear gene in plant cells. To answer this question, COs were designed to modify an ALS gene in tobacco and an inactivated transgene that was created by deletion of a single nucleotide of the gene encoding the green fluorescent protein (GFP).

MATERIALS AND METHODS

Maintenance of Tobacco Cell Suspension Cultures and Plant Transformation. Tobacco Nt-1 cell suspensions were maintained as shaker cultures (27°C, 200 rpm in a 250-ml flask) and transferred weekly to fresh suspension medium (CSM) containing Murashige and Skoog salts (GIBCO/BRL), 500 mg/liter Mes, 1 mg/liter thiamin, 100 mg/liter myo-inositol, 180 mg/liter KH_2PO_4, 2.21 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D), and 30 g/liter sucrose (pH 5.7). For solidified medium, 8 g/liter agar-agar (Sigma) was added before autoclaving. Cell suspensions maintained for biolistic delivery were subcultured weekly by transferring 1 ml of an established suspension culture into 49 ml of fresh liquid CSM.

Transgenic tobacco plants expressing a nontranslatable form of GFP under the control of the CaMV 35S promoter were generated by using a standard *Agrobacterium*-mediated plant transformation protocol (11).

CO Design, Synthesis, and Labeling. COs were designed based on the sequence of the tobacco ALS SuRA allele (GenBank accession no. X07644) and the sequence of GFP (12). CO ALS1 was designed with the Pro-196 codon CCA altered to contain a mismatch codon CAA (which encodes glutamine). A second CO (ALS2) was designed to substitute CTA (a leucine codon) at codon 196 of ALS SuRA (Fig. 1). COs were synthesized and purified according to ref. 3. The SuRA and SuRB alleles differ by only a single nucleotide in the region targeted by COs. A nonspecific CO (NSC1) was designed as a negative control by using the same general CO

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Abbreviations: CO(s), chimeric oligonucleotide(s); ALS, acetolactate synthase; GFP, green fluorescent protein.

A Commentary on this article begins on page 8321.

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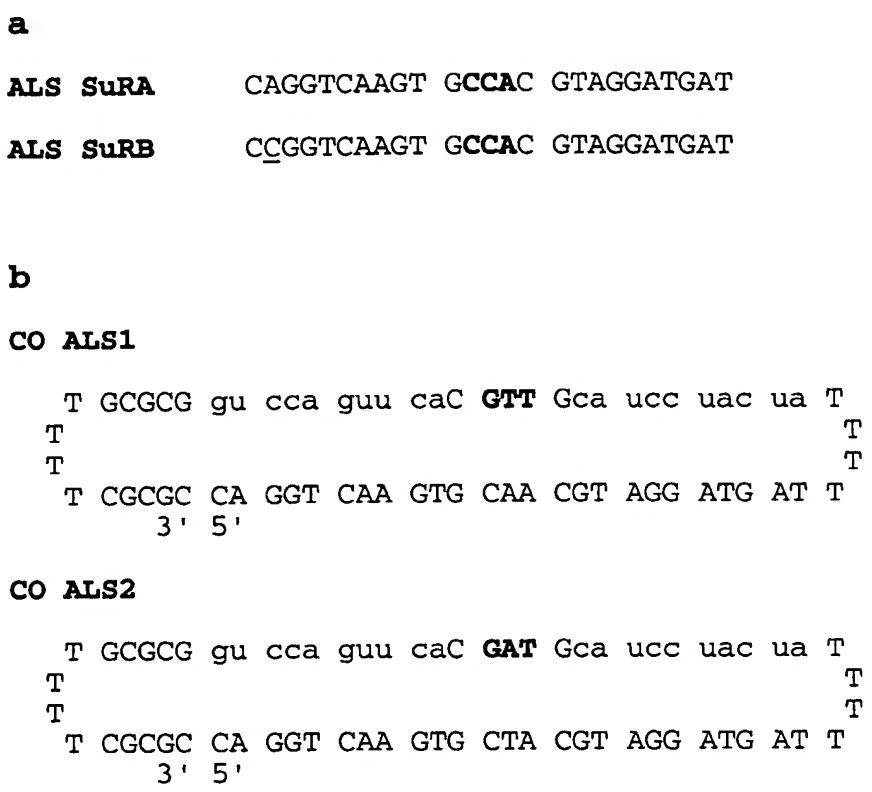

FIG. 1. Target sequence of ALS SuRA and SuRB alleles (*a*) and COs ALS-1 and ALS-2 (*b*). The single nucleotide difference between the ALS SuRA and SuRB alleles in the target region is underlined. Lowercase letters represent 2′-*O*-methyl-RNA residues. Bold letters represent the codon for Pro-196. DNA residues represented by uppercase letters.

structure as ALS1 and ALS2 but with a non-ALS-specific homology domain. A control oligonucleotide (ALSD) was designed as being identical in sequence to ALS1 but was composed only of DNA. The CO GFP1 was designed to correct a single base deletion in codon 6 of a mutant form of GFP (12).

Uptake studies were performed by using 1-μg aliquots of COs labeled with dUTP rhodamine by using terminal transferase as per the manufacturer's protocol (Promega).

Biolistic Delivery of COs. One milliliter (packed cell volume) of Nt-1 cell suspensions were subcultured onto plates containing solid CSM medium 3–5 days before microparticle bombardment. By using a helium-driven particle gun (Bio-Rad), COs were introduced to Nt-1 cells after precipitation onto 1-μm gold microcarriers (Bio-Rad). COs were precipitated onto microcarriers as follows. First, 35 μl of a particle suspension (60 mg of microcarriers per ml of 100% ethanol) was transferred to a 1.5-ml microcentrifuge tube, which was agitated on a vortex mixer. The following items were added in the order indicated: 40 μl of resuspendend CO (60 ng/μl water), 75 μl of ice-cold 2.5 M $CaCl_2$, and 75 μl of ice-cold 0.1 M spermidine. The tube was then mixed vigorously on a vortex mixer for 10 min at room temperature. The particles were allowed to settle for 10 min and were centrifuged at 11,750 × *g* for 30 sec. The supernatant was removed and the particles were resuspended in 50 μl of 100% ethanol. An aliquot of 10 μl of the resuspended particles was applied to each macroprojectile, which was used to bombard each plate once at 900 psi (1 psi = 6.89 kPa) with a gap distance (distance from power source to macroprojectile) of 1 cm and a target distance (distance from microprojectile launch site to target material) of 10 cm.

Selection of Herbicide-Resistant Cell Lines. Cells were transferred separately into 15-ml culture tubes containing 5 ml of liquid CSM 2 days after bombardment. The tubes were inverted several times to disperse cell clumps. Samples were then transferred to solid CSM medium containing 15 ppb chorsulfuron (DuPont). From ≈10–30 days after plating, actively growing cell masses were periodically selected and transferred to solid CSM containing 50 ppb chlorsulfuron. Three to four weeks later, actively growing cell masses were transferred to solid CSM containing 200 ppb chlorsulfuron. Cell lines that grew readily on medium containing 200 ppb of the herbicide were characterized at the molecular level.

Molecular Characterization of Cells Bombarded with COs ALS1 and ALS2. Genomic DNA was extracted from cell masses actively growing on selective medium. These DNAs were included in a PCR designed to preferentially amplify a 472-bp region of the ALS SuRA allele that included the targeted Pro-196. The PCR was completed with a Perkin–Elmer thermal cycler (model 480) by using the primers ALSprimer-1 (5′-GGGGTACCGGATTTCCCGGCGTTTG-3′) and ALSprimer-3 (5′-GTGGGGGGTGGGTGTCGGATCCCG-3′) with the following cycling conditions: 92°C 5 min, followed by 35 cycles of 92°C for 50 s, 63°C for 1 min, and 72°C for 1 min, ending with a 7-min extension at 72°C.

Amplification products were gel-purified by using a GeneClean kit (Bio 101) according to the supplier's recommendations. Purified PCR products were either directly sequenced on an ABI 310 automated sequence analyzer or sequenced after ligation into pGEM-T(easy) plasmid (Promega). Genomic DNAs from untreated tobacco Nt-1 cell suspensions were included as controls.

Reactivation of GFP. Transgenic tobacco plants expressing the mutant GFP transcripts were created and verified for the presence of the inactive gene by using Northern analysis. Tissues from these plants displayed no green fluorescent signal and were therefore used as targets for bombardment with the CO GFP1. After bombardment, the onset of green fluorescence was an indicator of restoration of an inactive gene, which

could only occur if the frameshift mutation in the transgene-encoding GFP was corrected.

RESULTS AND DISCUSSION

Introduction of Chimeric Oligonucleotides into Plant Cells. COs were fluorescently labeled in initial experiments to assist in the optimization of delivery into plant cells by electroporation (Fig. 2) and microparticle bombardment. As detected by using confocal UV microscopy, rhodamine-tagged COs were observed within plant cells, and were preferentially localized in the nucleus. The details supplied in the experimental protocols report our optimized precipitation and biolistic parameters for CO introduction into plant cells.

COs ALS1 and ALS2 were introduced into Nt-1 cells by microparticle bombardment. The following controls were also included in our experimental design: nonbombarded cells; cells bombarded with gold microprojectiles from a precipitation in which COs were excluded; cells bombarded with an oligonucleotide (ALSD) of DNA sequence identical to CO ALS1 synthesized without RNA residues (i.e., an all DNA oligonucleotide); and cells bombarded with a nonspecific CO (NSC1).

After CO introduction, putative conversions to herbicide resistance were identified as cell masses actively growing on selective medium (Fig. 3). Table 1 summarizes the results of three separate experiments. It has been demonstrated that spontaneous mutations in ALS can be recovered in tobacco cells in culture (7) and that herbicide resistance may arise from independent, different amino acid substitutions in the enzyme (8–10). Amino acid substitutions at codons other than Pro-196 of the tobacco ALS gene can confer resistance to both sulfonylurea (Glean) and imidizoline (Scepter) herbicides, whereas a mutation at codon 196 leads to selective resistance to Glean but not Scepter (9, 10). To eliminate possible spontaneous mutations at sites in the gene other than Pro-196, candidate cell lines were grown on medium containing 3 ppm imazaquin (negative selection) (Table 1).

By measuring growth of the Glean-resistant cells on Scepter, it was possible to determine further the ratio of Glean-resistant/Scepter-sensitive cells recovered. Mutation to this phenotype was 20-fold higher in the samples bombarded with either COs ALS-1 or ALS-2 when compared with the untreated controls. This finding suggests that codon 196 had been selectively mutated. To obtain more precise evidence that COs ALS1 and ALS-2 were causing a selective mutation rate

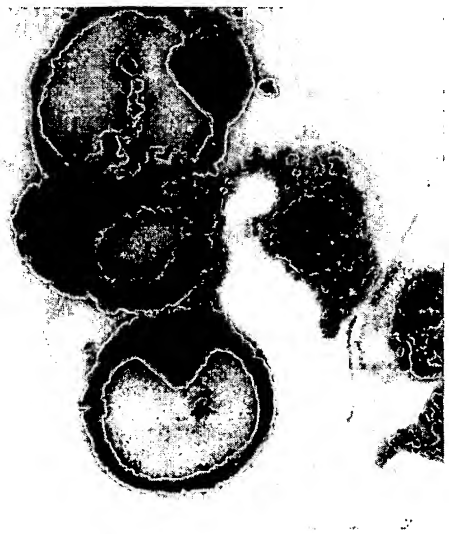

FIG. 2. Confocal photomicrograph demonstrating nuclear localization of rhodamine-tagged COs in tobacco Nt-1 cell protoplasts.

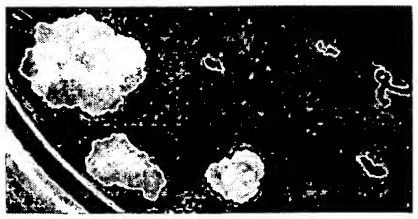

FIG. 3. Generation and selection of Glean-resistant Nt-1 cells grown on selective medium. Three-day-old plate cultures of Nt-1 cell suspension were used in ALS CO biolistic delivery experiments. Bombarded cell cultures were maintained on 15 ppb chlorsulfuron; putatively converted Nt-1 cell masses were transferred to medium containing 50 ppb chlorsulfon.

increase at codon 196, PCR products that amplify this region of the ALS sequence were generated and sequenced.

It is important to note that there are four possible locations in the tobacco genome at which codon 196 can be mutated. That is, in the allotetraploid genome, Pro-196 could be mutated in either copy of SuRA or SuRB. Fig. 4 shows an electropherogram of the DNA sequence analyses of PCR products from Glean^R/Scepter^S cell lines. If an individual ALS gene at one locus had been mutated, but not the other ALS loci, it would be expected that a dual peak would be found in the PCR product at the point of mutation. This was observed at the codon from amino acid 196 (see arrow); we concluded that at least one gene of the ALS multigene family had been altered. To confirm this observation, PCR products were ligated into the plasmid pGEM-T(easy), amplified, and individually sequenced. Cloned PCR products from the control cell lines demonstrate wild-type sequence in every instance, whereas 12–67% of the cloned PCR products generated from Glean^R/Scepter^S cell lines contained a modified base at the codon for amino acid 196. The fact that the cloned PCR products derived from the SuRB allele did not contain a mutation can be interpreted as evidence that the COs ALS1 and ALS2 are acting selectively at the SuRA allele.

Interestingly, the modified base in the Pro-196 codon was always found to be one nucleotide 5′ of the mismatch nucleotide of both COs. This heterotopic modification converted the Pro-196 CCA to a Thr-196 ACA (with CO ALS1) or Ser-196 TCA (CO ALS2) rather than the CAA or CTA as projected. The heterotopic modification of ALS by CO ALS2 had both ACA and TCA conversions. The phenomenon of a selective 5′-shift in mismatch "repair" (or, more correctly, the site of the observed mutation) may be indicative of the repair enzyme activities in plant cells under these experimental conditions. It has been previously reported that the formation of abasic sites may lead to nontargeted mutations in other systems (13, 14). For example, Bishop *et al.* (13) examined the miscoding properties of modified guanine residues bearing increasingly bulky O-6 substituents. Rat4 cells were transfected with plasmids carrying H-ras genes in which *O*-6-ethyl- and *O*-6-benzylguanine were substituted for the first or second

Table 1. Summary of ALS gene conversion experiment

Treatments	Plates, no.*	Glean^R/Scepter^S cell masses, no.†
Untreated	15	2 (0.1)
Microcarriers	15	1 (0.1)
CO NSC1	9	3 (0.3)
ALSD (DNA only)	9	2 (0.2)
CO ALS-1	9	21 (2.3)
CO ALS-2	9	19 (2.1)

*Number of plates used in experiment (One ml of packed cell volume bombardment).
†Number of Glean^R/Scepter^S cell masses obtained. Frequency per bombardment is shown in parentheses.

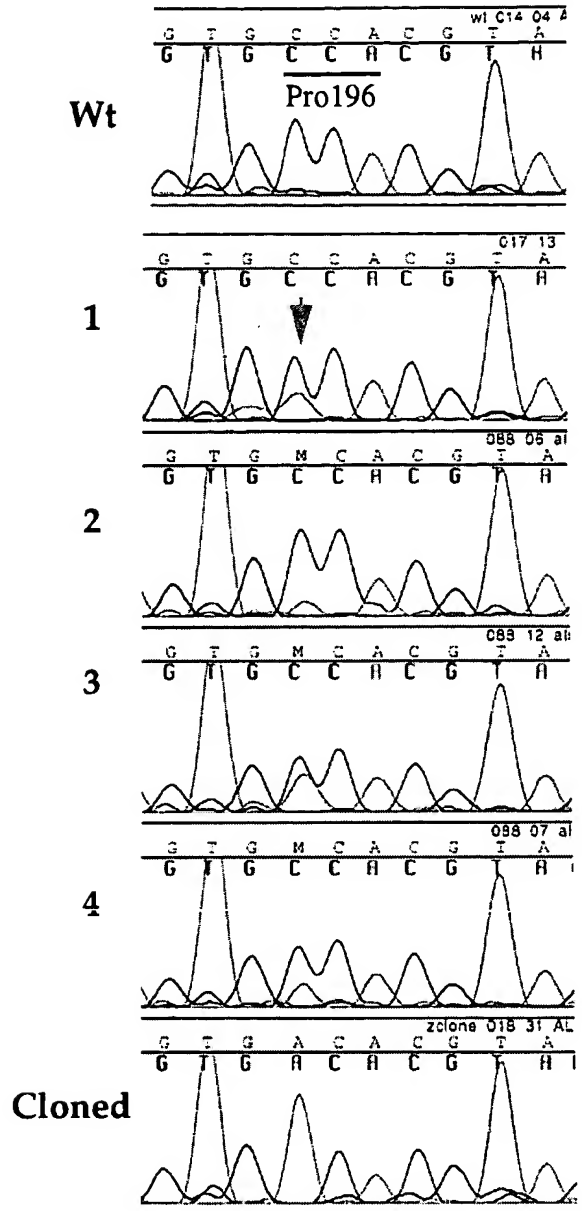

FIG. 4. Automated nucleotide sequence analyses of gene modification events. Electropherograms displaying the sequence of ALS-specific PCR products derived from DNA isolated from untreated Nt-1 cells (wt) and herbicide-resistant Nt-1 cells recovered as a result of an introduced CO (1–4) indicate an alteration in the native sequence for codon 196 (CCA). The conversion (C → A; arrow) in codon 196 (CCA) was detected (1–4) and is consistent with a targeted mutation induced by the CO. Because there are multiple copies of ALS alleles, modification of a single gene can confer herbicide resistance, but sequence analyses will indicate base composition heterogeneity at the revised position. To characterize individual species from the heterogeneous population of the ALS-specific PCR products derived from the DNA of herbicide resistant cells, shown in electropherograms 1–4, these products were cloned and sequenced. Sequence analysis of 1 of the 24 randomly selected clones, shown (Cloned), demonstrates the C → A conversion.

guanine residues of codon 12 (GGA). Their results demonstrate that either O-6-substituents induced "semitargeted" as well as targeted mutations. Semitargeted mutations were strictly G → A at the base 5′ to a position 2 adduct. The authors postulated that the mechanism for nontargeted mutations may be related to abasic site formation or to translesion DNA

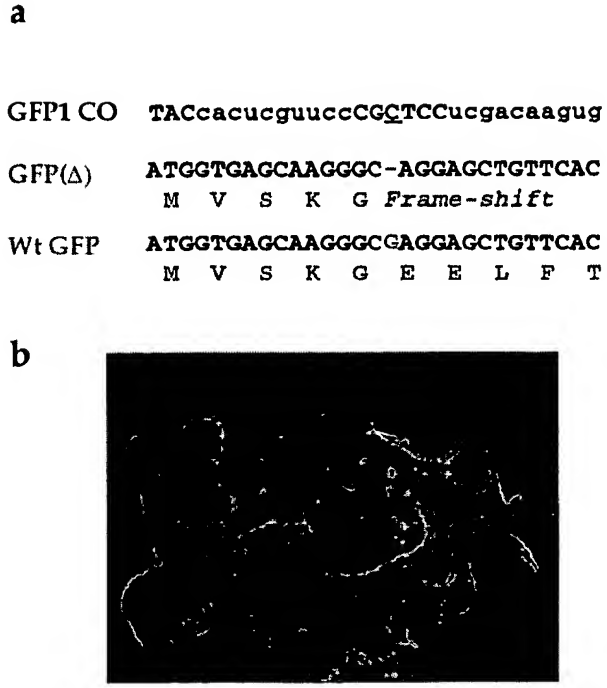

FIG. 5. Target sequence of the mutant GFP(Δ) and the GFP1 CO (*a*). Fluorescence photomicrograph of a putative conversion to the GFP phenotype in leaf callus from the tobacco GFP-mutant line Δ6 (*b*).

synthesis. In addition, Kamiya *et al.* (14) constructed c-Ha-ras genes with a true abasic site in codon 12. The ras genes were found to be activated in NIH 3T3 cells by a mutation at the modified site and, more frequently, at flanking positions.

Tobacco Nt-1 cells are allotetraploid with a genome of ≈4,000 megabase pairs (9). The ALS gene (SuRA allele) therefore is a target gene in a complex plant genome. CO bombardment resulted in herbicide-resistant cell lines that were shown to have nucleotide modifications at the codon encoding Pro-196. We have two lines of evidence demonstrating that the herbicide-resistant cells we recover do not arise from spontaneous mutations. First, in experiments using an equal number of Nt-1 cells, we have recovered 10- to 20-fold less herbicide-resistant cell lines after bombardment with uncoated gold microprojectiles, a nonspecific CO, or a "DNA-only" oligonucleotide. That is, the ALS COs increase mutation frequency by as much as a factor of 20. The fact that the increased mutation frequency is due to a site-specific mutation was verified by sequence analyses of ALS-specific PCR products (Fig. 4) amplified from the DNA of herbicide-resistant cells and indicates that these cells contain a base change specifically in the codon for Pro-196. PCR products subsequently cloned and sequenced confirm the presence of amplified DNAs with a modified Pro-196 codon. These modified cell lines have demonstrated genotypic stability by maintaining herbicide resistance over several months, and the modified Pro-196 codon can also be identified as a stable nucleotide change, by repeated PCR, cloning, and sequencing.

Our data of cell clumps/bombardment suggest the efficiency of base conversion at the ALS locus is up to two orders of magnitude higher than the controls, although the efficiency of CO-directed conversion was variable between experiments. These results are similar to the recent observations of CO-directed conversion in other systems (2, 3). Variation in efficiency may be caused by variation in CO delivery or cellular competency for conversion, which may vary with cell cycle or may be related to activity of DNA repair enzymes.

Further studies of other sequence targets may help to clarify the heterotopic nature of gene modification observed in this study. Interestingly, conversions directed by the ALS2 CO resulted in both C → T and C → A changes. Further characterization of plant DNA recombinase and repair enzymes may provide a better understanding of CO mode of action in plants.

Reactivation of an Inactive GFP Transgene. GFPs are a unique class of proteins involved in the bioluminescence of many jellyfish. In plants, GFP has been extensively used as a reporter for gene expression in both transient and stable expression systems (12). With the idea of using GFP as a model system to monitor conversion events in plant cells, we generated a mutant GFP expression vector that contained a single base pair deletion in the ORF that results in a frameshift mutation, thus preventing translation of the protein.

The CO GFP1 (designed to restore function to GFP) was introduced into the tobacco GFP-mutant line Δ6 by microparticle bombardment, and recovery of the GFP phenotype was observed by using fluorescence microscopy (Fig. 5). Putative conversion events were identified on the basis of restoration of the GFP phenotype. No GFP expression was observed with the nonspecific CO controls. GFP expression was observed over a period of 3–10 days postbombardment.

This successful reactivation of the GFP gene provides evidence that COs can successfully catalyze the insertion of a nucleotide in a nuclear transgene that we had chosen for its visible marker phenotype. It suggests this "nucleotide insertion technology" would also be applicable for introduction of a base into actively expressed coding sequences, thus rendering the targeted gene inactive by causing a frameshift mutation.

Unlike antisense or cosuppression strategies, directed gene knockouts would be of value in inactivating specific members of plant multigene families; as long as a nonhomologous sequence in the members of the family can be identified for use in designing a CO. In addition to gene silencing, this technology could be used to alter untranslated or nontranscribed regions of a gene.

SUMMARY

The experiments presented herein have shown two different types of induced mutation that are catalyzed in a site-specific means by COs designed to hybridize to a unique 25-base sequence in the nuclear genome. First, the mutational frequency for alteration of a specific codon in a gene can be enhanced by as much as 20-fold. We anticipate that this mutational efficiency will be increased when the mechanism controlling the CO-induced mutation process is better characterized. Second, the coding sequence of a nuclear gene can be selectively altered by the insertion of an additional nucleotide; this is catalyzed specifically by the CO designed for the unique DNA sequence where the base was inserted, because the activation of the GFP was never observed in the absence of the appropriate CO.

A further experiment to combine the above-mentioned types of mutations can now be contemplated. If two different COs were both delivered into plant cells, it is hypothetically possible that a selectable mutation could be created in a plant cell together with a second mutation that would cause a frameshift inactivation of an unlinked gene. That is, dual mutations might be created in which one is known with respect to function, but the second could be created to explore the cellular consequence of loss of function of a unique coding sequence. If perfected, this approach could have great value in functional genomics. The approach could also have more immediate value in causing the inactivation or alteration of endogenous plant genes wherein the alteration could increase crop value.

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Plant Biology

Targeted manipulation of maize genes *in vivo* using chimeric RNA/DNA oligonucleotides

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ABSTRACT **Site-specific heritable mutations in maize genes were engineered by introducing chimeric RNA/DNA oligonucleotides. Two independent targets within the endogenous maize acetohydroxyacid synthase gene sequence were modified in a site-specific fashion, thereby conferring resistance to either imidazolinone or sulfonylurea herbicides. Similarly, an engineered green fluorescence protein transgene was site-specifically modified *in vivo*. Expression of the introduced inactive green fluorescence protein was restored, and plants containing the modified transgene were regenerated. Progeny analysis indicated Mendelian transmission of the converted transgene. The efficiency of gene conversion mediated by chimeric oligonucleotides in maize was estimated as 10^{-4}, which is 1–3 orders of magnitude higher than frequencies reported for gene targeting by homologous recombination in plants. The heritable changes in maize genes engineered by this approach create opportunities for basic studies of plant gene function and agricultural trait manipulation and also provide a system for studying mismatch repair mechanisms in maize.**

Site-directed manipulation of chromosomal genes has become the method of choice for determining gene function in bacteria, yeast, and mammalian cells. The primary methods used in site-directed gene manipulation rely on gene replacement via homologous recombination using an appropriately designed gene targeting vector (1). In plant cells, gene targeting has been limited by the low frequency of homologous recombination (1, 2). Homologous DNA fragments are randomly integrated into the genome at a much higher frequency (2–7). Even with recent improvements in transformation and selection conditions, the reported frequency of gene targeting in plant cells is still about one event in 10^5–10^7 targeted cells (8–10).

Procedures being developed for mammalian gene therapy provide potential alternatives for gene targeting in plants. One such example is an approach using chimeric RNA/DNA oligonucleotides (ONDs) (11, 12). In mammalian cells, chimeric ONDs that contain both DNA/DNA and RNA/DNA duplex regions with homology to a target locus are capable of specifically correcting mutations at a high frequency in both episomal and chromosomal target genes (11, 12). Gene conversion requires RNA/DNA duplex regions in the targeting ONDs and can occur in 30–40% of recipient cells (12–14), a frequency that is several orders of magnitude higher than gene targeting via homologous recombination. Targeting in mammalian cells with chimeric ONDs is also highly specific, because base alteration was found not to occur in related genes with mismatches in the sequence spanned by the RNA region of the ONDs (12). To date, however, only a few examples of chimeric OND-based gene targeting experiments have been reported in plant systems, and no significant data have been published. Furthermore, the heritability of targeting events reported in mammalian cells has not been vigorously investigated (11–16). For practical utility in plants, stable transmission of modified genetic traits to progeny is a requirement.

Mutations induced by chimeric RNA/DNA ONDs generally have involved alteration of 1–2 bp in the target site, which is adequate for many applications such as site-specific mutagenesis, gene knockouts, and allelic replacements. We report here targeted modification of an endogenous gene and an engineered transgene in maize by using chimeric RNA/DNA ONDs. Our results demonstrate that maize genes can be modified specifically and efficiently by chimeric ONDs and suggest that reverse genetics and engineering of endogenous genes in commercially important crops will be feasible by using this approach.

MATERIALS AND METHODS

Transformation Vectors. Transformation vectors were constructed as controls by using standard gene cloning methods. The plasmid pPHP10247 contains the *in vitro* mutagenized maize acetohydroxyacid synthase (AHAS) 108 gene encoding the Ser-621–Asn mutant form (17). The gene is flanked by the maize ubiquitin-1 promoter (18) and the nopaline synthase polyadenylation signal (19). The plasmid pPHP12322 contains the Pro-165–Ala mutant form of maize AHAS108 (20). Both plasmids have pUC-derived backbones. The plasmid pPHP3528 contains the *Streptomyces hygroscopicus bar* gene (21) driven by the maize ubiquitin-1 promoter.

For the transgene target we created a translational fusion between phosphinothricin-*N*-acetyltransferase (PAT), a gene product conferring resistance to bialophos (22), and the green fluorescence protein (GFP) (23). The fusion was created by cloning a 3′ *Bgl*II site in PAT to a 5′ flanking *Bam*HI site on GFP. By site-directed mutagenesis (MORPH kit, 5 Prime→3 Prime), the start codon (ATG) from GFP was removed, and a native PAT termination codon (TGA) was inserted in the junction of PAT/GFP. The plasmid PHP11129 contains the coding sequence for the PAT/TGA/GFP fusion target sequence, the maize ubiquitin-1 promoter, and the pinII terminator in a superbinary vector pSB1 suitable for *Agrobacterium*-mediated transformation (24). The plasmid PHP10699 is a positive control of the fusion without the TGA codon.

Cell Culture, Transformation, and Selection. Cultured maize HiII (25) or Black Mexican Sweet (BMS) (26) cells, as well as immature embryos from GS-3 (HiII equivalent), were used for transformation experiments. Transformation medi-

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Abbreviations: AHAS, acetohydroxyacid synthase; OND, oligonucleotide; PAT, phosphinothricin-*N*-acetyltransferase; GFP, green fluorescence protein; BMS, Black Mexican Sweet.

A Commentary on this article begins on page 8321.

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ated by particle bombardment was performed according to Tomes *et al.* (27). *Agrobacterium*-mediated transformation was performed according to Ishida *et al.* (24).

Transformed cells were plated on solid culture medium containing either 0.7 μM imazethapyr (AC263, 499, or Pursuit, technical grade, American Cyanamid) for AHAS621 or 20 ppb chlorsulfuron (Glean, technical grade, DuPont) for AHAS165. Putative events were identified 4–6 weeks after bombardment and subsequently selected on fresh media containing 1.0–2.0 μM imazethapyr or 50 ppb chlorsulfuron.

The transgenic positive control lines were established by particle bombardment-mediated transformation of HiII cells with either pPHP10247 (AHAS621) or pPHP12322 (AHAS165) together with pPHP3528. Transformants expressing the *bar* gene were selected on media containing 3 mg/liter of bialaphos (Meiji Seika, Tokyo), and further selected on imazethapyr or chlorsulfuron. These transgenic events served solely as positive controls for imazethapyr or chlorsulfuron selection testing in culture and were not advanced for plant regeneration.

Stable lines with the PAT/GFP transgene were established via *Agrobacterium*-mediated transformation. Transformed immature embryos were selected on media containing 3 mg/liter of bialaphos. Plants were regenerated from HiII embyogenic callus containing verified converted PAT/GFP transgene according to Register *et al.* (28). Developing T_0 plantlets were transferred to soil and grown to maturity in the greenhouse. After pollination with HiII pollen, the T_1 seeds were collected. Forty seeds were germinated for progeny segregation analysis.

OND Synthesis, Labeling, and Plant Nuclease Resistance. Chimeric RNA/DNA ONDs were synthesized and purified according to ref. 11. Chimeric OND SC2 (12) was 3′ end-labeled with tetramethylrhodamine-6-dUTP (Boehringer Mannheim) by using terminal transferase according to the manufacturer's instructions.

Whole-cell extract was prepared from maize BMS cells by using a Bionebulizer (Glas-Col, Terre Haute, IN). Double-strand DNA, 2′-*O*-methyl-RNA, DNA/RNA hybrid, and RNA/DNA chimera, with similar length and secondary structure, were labeled with ^{32}P by using T4 polynucleotide kinase. Samples with the same amount of radioactivity were incubated with whole-cell extract at 17°C for 90 min. Controls included incubation in nuclease-free water and whole-cell extract inactivated at 65°C for 5–10 min. Results were examined by 12% PAGE and autoradiography. The percentages of intact OND in each sample were quantified from the autoradiogram by using ALPHAEASE software (Alpha Innotech, San Leandro, CA).

Chimeric OND Delivery. Chimeric ONDs were delivered to plant cells by particle bombardment. Briefly, onion epidermis was freshly prepared before bombardment. Cultured maize HiII or BMS cells were suspended in liquid N6 medium and then plated on a VWR Scientific glass fiber filter. Chimeric ONDs (0.4 μg) were coprecipitated with 15 μl of 2.5 mM $CaCl_2$ and 5 μl of 0.1 M spermidine onto 25 μg of 1.0-μm gold particles. Microprojectile bombardment was performed by using a Bio-Rad PDS-1000 He particle delivery system.

Fluorescent Microscopy. The *in vivo* fate of the rhodamine-labeled chimeric ONDs was monitored by using a Leica DM RB microscope with filter 41002b (Chroma Technology, Brattleboro, VT). Images were recorded by a CH350 charge-coupled device camera (Photometrics, Tucson, AZ). Superimposed images were processed by using Adobe Photoshop 4.0 (Mountain View, CA). Green fluorescence from GFP-expressing cells was surveyed by using a Leica MD-10 epifluorescence microscope with a Leica GFP filter set (10446093) 4 days after transformation. Images were recorded on Fujichrome Sensia film (ASA400).

PCR Amplification and Sequence Analysis. Target sequences were amplified from the extracted genomic DNA of putative events by *Pwo* polymerase (Boehringer Mannheim), with 30 cycles of 35 s at 95°C, 35 s at 60°C, and 35 s at 72°C. For the AHAS621 target, primers common to both AHAS108 and AHAS109 were designed as 5′-GCAGTGGGACAGGTTC-TAT (PHN21971) and 5′-AGTCCTGCCATCACCATCCA (PHN21972). For the AHAS165 target, the following primers were used: 5′-ACCCGCTCCCCCGTCAT (PHN21973) and 5′-ATCTGCTGCTGGATGTCCTTGG (PHN21974). For the PAT/GFP target, primers used were: 5′-CGCAACGCCTAC-GACTGGA (PHN21976) and 5′-TGATGCCGTTCTTCT-GCTTGTC (PHN21978). PCR fragments were purified and either cloned or directly sequenced in both directions on an Applied Biosystems ABI377 automated sequencer.

Restriction Fragment Length Polymorphism Analysis and Cloning. PCR fragments were digested with excess *Bfa*I (New England BioLabs) and analyzed by electrophoresis on gels containing 2% metaphor and 1.5% Seakem LE agarose (FMC) by using 1× Tris-borate EDTA. Undigested fragments were extracted and purified from gel slices by using a QIAquick gel extraction kit (Qiagen, Chatsworth, CA) and subcloned into the cloning vector pCR2.1-TOPO or pCR-Blunt (Invitrogen). Vectors containing subcloned fragment were transformed into Invitrogen's competent *Escherichia coli* One-Shot Top10 cells. Cloned fragments were sequenced by using M13 forward and reverse primers.

RESULTS

Nuclease Resistance and *in Vivo* Fate of Chimeric ONDs. First, we examined the stability of the radioactively labeled chimeric RNA/DNA ONDs in maize whole-cell extract. Quantitative analysis of the autoradiogram indicated that approximately 40–50% of chimeric ONDs remained intact after 90 min of incubation. To examine their fate *in vivo*, a rhodamine-labeled chimeric OND SC2 was bombarded into onion epidermis and BMS maize suspension culture cells. After bombardment, cells were rinsed with liquid culture medium and examined by fluorescence microscopy over time. Other than a diffuse signal in the cytoplasm, rhodamine fluorescence was localized mainly in nuclei and occasionally associated with gold

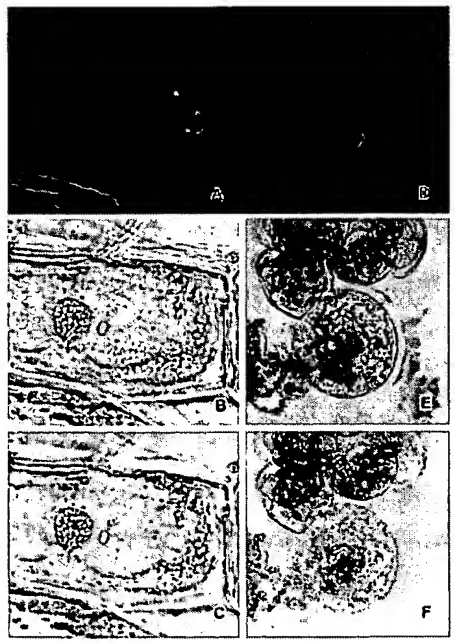

FIG. 1. Nuclear localization of rhodamine-labeled chimeric ONDs in onion epidermal cells (*A–C*) and maize BMS cells (*D–F*) 1 hr after bombardment, illustrated by rhodamine signal (*Top*), cellular organization (*Middle*), and their superimposed images (*Bottom*).

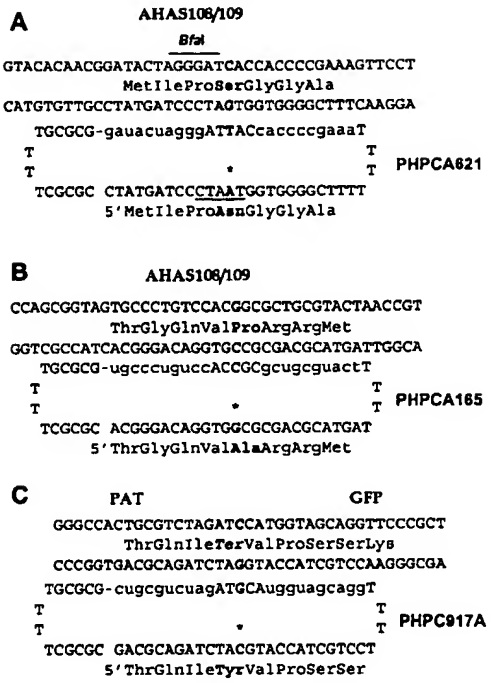

FIG. 2. Chimeric ONDs and target sequences of (*A*) AHAS Ser-621–Asn, (*B*) AHAS Pro-165–Ala, and (*C*) Ubi::PAT/GFP fusion Ter-996–Tyr. DNA residues are indicated in uppercase, and the modified RNA residues are shown in lowercase. Nucleotides in bold differ between the target sequence and chimeric OND. * indicates the nucleotide that should be introduced into the target sequence. The overscored sequence highlights the *Bfa*I restriction site and the underlined sequence indicates the site after sequence modification.

particles within cells (Fig. 1). We found that chimeric ONDs accumulate preferentially in the nuclei of these plant cells within 1 hr after bombardment. At 24 hr after bombardment, the rhodamine fluorescence was either very weak or no longer visible.

Conversion of Maize Endogenous *AHAS*. The endogenous gene target we chose for modification encodes AHAS (E.C. 4.1.3.18), the first enzyme in the biosynthetic pathway of branched chain amino acids. It is the target of imidazolinone and sulfonylurea herbicides (29, 30), and several mutations are known that confer resistance to these chemicals.

Maize AHAS Ser-621 corresponds to AHAS Ser-653 in *Arabidopsis* (17). A dominant single point mutation results in an amino acid substitution from Ser (AGT) to Asn (AAT) at the carboxyl terminal end of the mature AHAS, thus conferring resistance to the imidazolinone herbicide family. Two AHAS genes, *AHAS108* and *AHAS109*, previously have been reported in maize (31). In the maize HiII cell line, there are two copies of *AHAS108* and five copies of *AHAS109*, both of which are identical in nucleotide sequence at the target site (T.Z., M. Rudert, and C.L.B., unpublished data). Chimeric OND PHPCA621 was designed to modify Ser-621 in both AHAS108 and AHAS109, while simultaneously removing a *Bfa*I site (Fig. 2).

Maize AHAS Pro-165 corresponds to the AHAS Pro-196 site in tobacco (20). Various dominant mutations at this position lead to sulfonylurea herbicide resistance in many species (32). Chimeric OND PHPCA165 was designed to introduce a Pro-165–Ala mutation through a single nucleotide substitution from a CCG to a GCG in either *AHAS108* and *AHAS109*, because the two sequences are identical at this target site. No alteration of restriction sites was associated with this change (Fig. 2).

These chimeric ONDs were introduced independently into maize HiII and BMS cells by microprojectile bombardment. Resistant calli were selected on imazethapyr or chlorsulfuron, and the results are summarized in Table 1. In various negative controls (see below), five spontaneous mutants resistant to imazethapyr were identified from 86 plates, each plate containing approximately 10^6 cells, and one spontaneous mutant resistant to chlorsulfuron was selected from 50 plates. Thus the frequencies of spontaneous mutations conferring imazethapyr or chlorsulfuron resistance were 10^{-7}–10^{-8}.

Fragments containing the targeted region of *AHAS* from herbicide-resistant calli were amplified by PCR for sequence analysis. For AHAS621, mutant alleles first were identified by restriction fragment length polymorphism using *Bfa*I. Fragments containing the wild-type allele produced restriction fragments of 244 bp and 44 bp after digestion, whereas fragments with the mutant allele remained unrestricted by *Bfa*I (Fig. 3 *A* and *B*). In the positive control callus lines, where multiple copies of mutant *AHAS* were introduced by bombardment (data not shown), there are approximately equal amounts of restricted and unrestricted fragments, indicating multiple copies of endogenous wild-type *AHAS* genes. Among the fragments amplified from two herbicide-resistant calli obtained after chimeric OND treatment, a band corresponding to the unrestricted *Bfa*I fragment was clearly present (Fig. 3*B*). However, the restricted fragments were still prevalent, indicating that only a proportion of endogenous target sites were converted. The unrestricted fragments from both events were isolated and cloned. Sequence analysis of these clones indicated that 34 of 40 clones contain the change predicted by the specific chimeric OND (G to A, Fig. 3*C*). Unexpectedly, the remaining six clones contain three alternative mutations in adjacent bases (Table 2). Each of the three alternative mutations also resulted in loss of the *Bfa*I restriction site in the target sequence.

Sequence alterations from 16 additional herbicide-resistant calli and three controls were examined by direct sequencing of fragments amplified by PCR. Mutated target sequences were observed in fragments amplified from 11 of 16 chimeric OND-derived events and two positive control calli, but not in

Table 1. Summary of gene conversion experiments

Target	Cell type	Plates bombarded	Total cells receiving oligos*	Putative events selected	Events analyzed†	Confirmed mutation‡	Predicted conversion frequency§
AHAS621	HiII	130	2×10^5	40	18	13	1.4×10^{-4}
AHAS165	BMS	86	9×10^4	29	11	9	1.0×10^{-4}
PAT/GFP (T_0)	HiII	48	5×10^4	11	1	1	1.5×10^{-4}
PAT/GFP (T_1)	HiII	89	9×10^4	139	N/D	N/D	1.1×10^{-3}

*Total cells receiving chimeric ONDs were estimated by transient expression of GFP using bombardment of pPHP10699.
†Events were selected by their herbicide resistance or GFP phenotypes and analyzed by direct sequencing of PCR products or, where applicable, by restriction fragment length polymorphism and cloning.
‡Mutations induced by chimeric ONDs include those with the desired base change at the target site, as well as base changes at positions adjacent to the target sites (see Table 2).
§Predicted conversion frequency = (putative events selected × molecular confirmation rate)/total cells receiving chimeric ONDs.

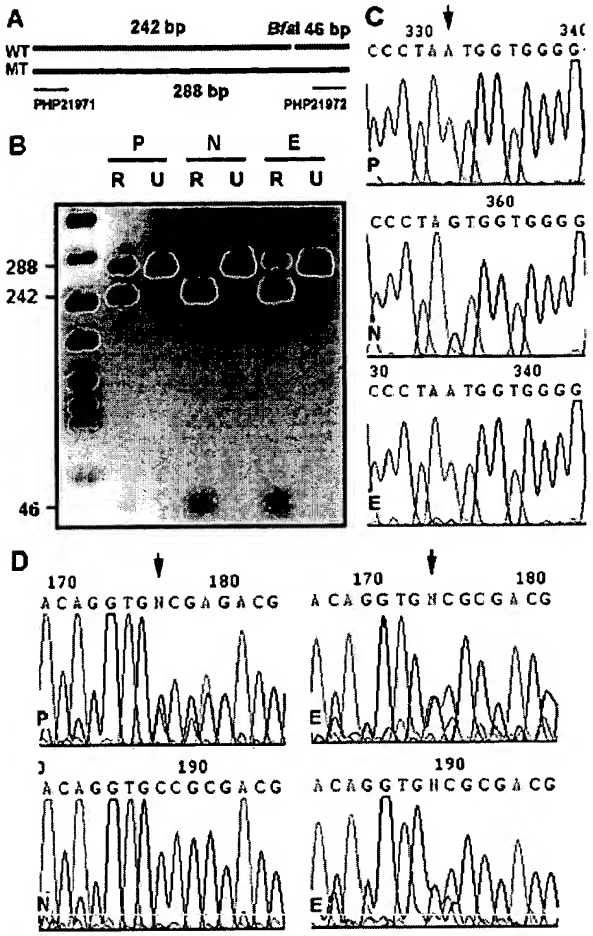

FIG. 3. AHAS621 conversion examined by restriction fragment length polymorphism and sequence analysis. (*A*) A map of the amplified AHAS621 target sequence from both wild-type and mutant alleles indicates the positions of PCR primers and the *Bfa*I restriction site. (*B*) Polymorphism of wild-type and mutant alleles in target PCR fragments from positive control (P), negative control (N), and a representative event (E) before (U) and after *Bfa*I restriction (R). (*C*) Sequence comparison of cloned AHAS621 alleles from the above samples. (*D*) Sequence comparison of cloned AHAS165 alleles from a positive control event (P), a negative control event (N), and two events with the predicted nucleotide conversion (E). Sequences were generated directly from PCR-amplified DNA from maize tissues. Because multiple AHAS genes exist in maize, as expected, both unconverted wild-type and converted mutant alleles are present in the events, as represented by the two overlapping peaks and the N nucleotide designation in the chromatograms.

wild-type callus. Thus, through direct sequencing of PCR products, 69% (11/16) of herbicide-resistant calli resulting from PHPCA621 treatment exhibited mutations at the target site.

For AHAS165, PCR fragments from target regions of resistant calli and a transgenic positive control line were sequenced directly. In the positive control callus expressing a Pro-165–Ala mutant form of maize AHAS from the ubiquitin promoter, the predicted change was detected from the chromatograms. The predicted conversion (Pro-165–Ala) also was detected from two of the chlorsulfuron-resistant calli analyzed (Fig. 3*D*). However, in a large proportion of the resistant calli, a T rather than the expected G was introduced at the predicted position, which resulted in a Pro→Ser conversion (Table 2). Nevertheless, because a substitution of Pro-165 with various amino acids will confer chlorsulfuron resistance, the mutations induced by PHPCA165 resulted in the desired phenotype.

No mutations were detected at the target position in herbicide-resistant calli arising by spontaneous mutation or in various negative controls, and no other mutations within 800 bp of surrounding sequence were found in any of the clones derived from the manipulated cells. The negative controls included: (*i*) unbombarded cells, (*ii*) cells bombarded with gold particles only, (*iii*) cells bombarded with PHPC917A chimeric OND that contains no homologous sequence to the AHAS target sites, and (*iv*) cells bombarded with a DNA-only version of PHPCA621.

Conversion of a *PAT/GFP* Transgene. The engineered transgene we used in this study is a stably integrated *PAT/GFP* fusion with a termination codon between the two genes, which prevents translation of the GFP protein. A chimeric OND (PHPC917A) was designed to replace G with C at nucleotide position 2990 (Fig. 3*B*), thereby eliminating the termination codon and allowing for expression of GFP as part of the PAT/GFP fusion protein. By using this scheme, we expected that modified cells should be identifiable by GFP fluorescence without chemical selection.

Two HiII transformants containing the *PAT/GFP* fusion gene were established by selection on bialaphos after *Agrobacterium*-mediated transformation. No GFP expressing cells were observed in either cell line (data not shown). Four days after introducing PHPC917A, GFP-expressing cells were identified in each cell line by fluorescence microscopy. In initial experiments using recipient cell lines that had been maintained in culture for 10 months, 11 GFP-positive events were detected in 48 bombardment plates. Subsequent experiments used freshly initiated cell lines derived from T_1 embryos of plants regenerated from the initial transformants; with introduction of PHPC917A, the frequency of GFP-positive cells was approximately 10-fold higher (Table 1). No GFP-positive cells were observed from various negative controls including: (*i*) unbombarded cells, (*ii*) cells bombarded with gold particles only, (*iii*) cells bombarded with a DNA version of PHPC917A, (*iv*) cells bombarded with nonspecific chimeric OND PHPCA621, and (*v*) wild-type cells bombarded with PHPC917A.

Table 2. Summary of mutations induced by chimeric ONDs

Target	Predicted change		Observed change		Frequency of sequence observed	
	Nucleotide	Amino acid	Nucleotide	Amino acid	Clones*	PCR fragments
AHAS621	AGT → AAT	Ser-621–Asn	AGT → AAT	Ser-621–Asn	34/40	13/16
			AGT → GGT	Ser-621–Gly	3/40†	0/16
			CCT → CCC	Pro-620–Pro	2/40†	0/16
			CCT → CAT	Pro-620–His	1/40†	0/16
AHAS165	CCG → GCG	Pro-165–Ala	CCG → GCG	Pro-165–Ala	–	2/12
			CCG → TCG	Pro-165–Ser	–	7/12
			CCG → ACG	Pro-165–Thr	–	1/12
PAT/GFP	TAG → TAC	Ter-996–Tyr	TAG → TAT	Ter-996–Tyr	–	1/1

*Clones were analyzed from two independent events, as described in text.
†Frequency enriched by *Bfa*I restriction.

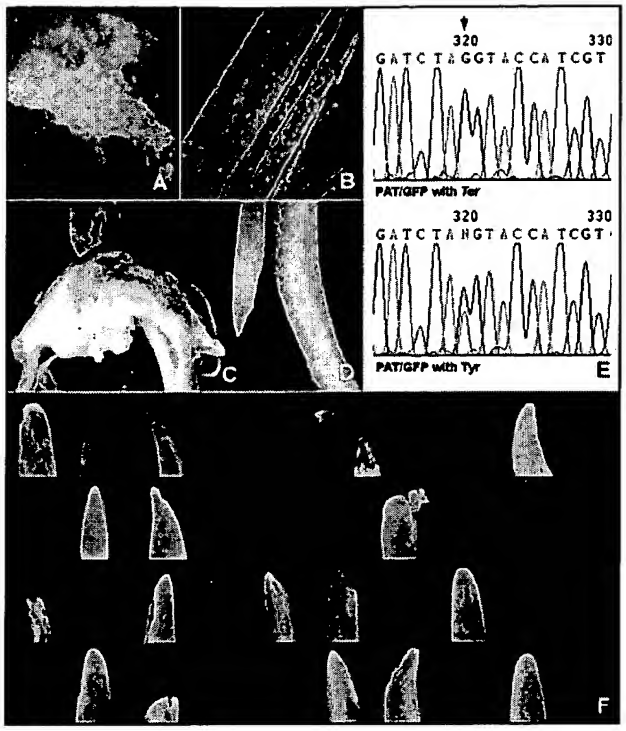

FIG. 4. GFP expression in T_0 callus (*A*), leaves (*B*), germinating embryo (*C*), and roots (*D*) after PAT/GFP conversion. (*E*) Chromatogram of the junction region of the targeted PAT/GFP showing sequence obtained directly from PCR amplification. (*F*) T_1 progeny segregation. In seedlings with GFP expression, strong green fluorescence was detected in the coleoptiles that lack chlorophyll. In contrast, coleoptiles in seedlings without GFP expression were semitransparent under the excitation and emission spectra used, and leaf chlorophyll autofluorescence could be seen through coleoptile tissue.

GFP-positive cell clusters (Fig. 4*A*) were excised and transferred to appropriate media for plant regeneration. GFP fluorescence was clearly seen in regenerating T_0 seedlings (Fig. 4 *B–D*). Sequence analysis of one of the T_0 convertants indicated replacement of the termination codon by a tyrosine codon. However, a T rather than the predicted C residue was found at the correct nucleotide position 2990 (Fig. 4*E*). The T_0 plants were fertile and produced viable seeds. Strong green fluorescence was observed from various tissues of T_1 seedlings, except in leaves where GFP fluorescence was masked by chlorophyll autofluorescence (data not shown). Analysis of 40 T_1 progeny indicated that 18 seedlings expressed GFP, consistent with a 1:1 Mendelian transmission of the modified PAT/GFP transgene (Fig. 4*F*).

DISCUSSION

Our results demonstrate that genes in maize can be modified at the nucleotide level with a high degree of precision by using chimeric RNA/DNA ONDs. Although chimeric ONDs with sequences identical to the target were not tested in this study, previous work in mammalian cells has shown that such ONDs apparently are not mutagenic (11, 12).

The overall frequencies of site-specific targeting by chimeric ONDs as reported here (10^{-4}, Table 1) are 2–3 orders of magnitude higher than frequencies of spontaneous mutation (10^{-7}–10^{-8}), and gene targeting by homologous recombination (10^{-5}–10^{-7}) in plant cells (2). However, the frequencies observed in plants are up to 3 orders of magnitude lower than the frequencies reported for chimeric OND-mediated nucleotide conversion in mammalian cells, depending on different target cell lines used (12, 14, 16). One explanation is that the frequency we observed might represent a conservative estimate because it is based primarily on events that survived chemical selection. Observed targeting frequencies using the PAT/GFP fusion target were higher, especially when healthy freshly initiated callus was used (Table 2). Although chemical selection provides a useful means for recovery of cells with the desired phenotype after targeting, some targeted cells may undergo cell cycle arrest as a result of DNA damage/repair (33, 34), and thus may not be easily recovered as colonies when additional stress is imposed. Other factors responsible for the different frequencies may involve experimental variables, such as method of delivery (bombardment vs. lipofection), or differences between mammalian and plant cells in the efficiencies of homologous pairing, strand transfer, or mismatch repair.

In addition to the predicted nucleotide conversions obtained in our studies, different mutated nucleotides were recovered in several cases from individual herbicide-resistant calli. However, it is not clear from our analysis whether any of the unexpected sequence changes result in the observed herbicide resistance. Similarly, it is not clear which of the multiple endogenous AHAS genes were mutated or if all cells in each callus contained each of the mutated AHAS forms detected by our analysis. It is possible that the diversity of mutations may result from decreased fidelity of the mismatch repair machinery in maize, as compared with mammalian cells. Error-prone mismatch repair may somehow be activated by specific sequences in the target region or by the affinity of the repair machinery for mismatched heteroduplex involving DNA and 2′-*O*-methyl RNA. At the very least, our data indicate that chimeric RNA/DNA ONDs could be used to assay the poorly characterized mismatch repair pathways in plants.

The proliferation of targeted cells and heritable transmission of targeted genes shown here suggest that chimeric RNA/DNA ONDs will be useful for applications in plants such as reverse genetics and crop improvement. With previous gene therapy applications, altered genes generally were not transmitted even through mitosis, because the targeted cells, such as lymphoblasts and hepatoma cells, were terminally differentiated (12–15), although a recent study demonstrated the mitotic stability of a corrected gene in melanocytes (16). The present system should provide opportunities for studying gene function through the ability to create targeted gene modifications and for the generation of novel traits in plants, without needing to introduce foreign genes.

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Chimeric RNA/DNA Oligonucleotide-Based Site-Specific Modification of the Tobacco Acetolactate Syntase Gene

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Single amino acid substitutions at either of two crucial positions in acetolactate synthase (ALS) result in a chlorsulfuron-insensitive form of this enzyme and, as a consequence, a herbicide-resistant phenotype. Here, we describe the successful in vivo targeting of endogenous tobacco (*Nicotiana tabacum*) ALS genes using chimeric RNA/DNA and all-DNA oligonucleotides at two different locations. Similar number of conversion events with two different chimeras indicates the absence of restricting influence of genomic target sequence on the gene repair in tobacco. Chlorsulfuron-resistant plants were regenerated from calli after mesophyll protoplast electroporation or leaf tissue particle bombardment with these specifically constructed chimeras. Sequence analysis and enzyme assays proved the resulting alterations to ALS at both DNA and protein levels. Furthermore, foliar application of chlorsulfuron confirmed the development of resistant phenotypes. Lines with proline-196-alanine, threonine, glutamine, or serine substitutions or with tryptophan-573-leucine substitutions were highly resistant at both cellular and whole plant levels, whereas lines with proline-196-leucine substitutions were less resistant. The stability of these modifications was demonstrated by the continuous growth of calli on chlorsulfuron-containing medium and by the transmission of herbicide resistance to progeny in a Mendelian manner. Ability of haploid state to promote chimera-mediated conversions is discussed.

The glut of sequence information now available for many organisms demands the application of reliable reverse genetics techniques to associate genes with their functions. Several reverse genetics strategies have been developed and are currently used in plants with different degrees of success. Some of them, like T-DNA or transposon insertional mutagenesis, fast neutron deletion mutagenesis, and the targeting of induced local lesions in genomes, are based on random processes (integration or mutation) and the subsequent application of various methods to identify desirable mutants. In many non-plant model species, homologous recombination is used to target genes for mutagenesis. Although gene targeting has been achieved in plants (for ectopic and endogenous genes), it is still far from routine. Low frequency of homologous recombination and inaccurate integration are basic barriers that must be overcome to make gene targeting work in higher plants (Morton and Hooykaas, 1995; Mengiste and Paszkowski, 1999).

A new strategy, chimera-directed gene alteration, was developed to correct or induce site-specific point mutations in mammalian cells and has been applied recently to animals and yeast (Yoon et al., 1996; Alexeev et al., 2000; Bartlett et al., 2000). This gene targeting strategy is based on the action of chimeric RNA/DNA oligonucleotides (chimeras; Yoon et al., 1996). Chimeric oligonucleotides are self-complementary and produce double-hairpin structures. In this duplex conformation, two complementary strands can be distinguished: a chimeric strand consisting of an interposed DNA fragment (5 bp) and two stretches of RNA (10 bp) flanking this region, and another strand that is composed only of DNA. The specific structure of this vector (two thymidine hairpins, a "GC" clamp at the 3′ end, and 2′-*O*-methylated RNA residues) makes it stable within cells. To target a gene, a chimera must be identical to the gene with the exception of a single nucleotide. This produces a mismatched base that is presumably recognized by endogenous repair machinery. The precise mechanism of this conversion is unknown, but it is believed that it is based on a two-step process: homologous pairing that results in the formation of a double D-loop structure and subsequent endogenous repair activity. From recent studies, it appears that the chimeric strand provides stability to the intermediate joint molecule, whereas the DNA strand is used by endogenous repair machinery as a template for correction (Gamper et al., 2000a). The possibility of using a chimeric oligonucleotide to promote site-specific alterations was shown for the first time in episomal DNA (Yoon et al., 1996). Later, it was successfully targeted to chromosomal sequence in mammalian cells and animal models (Kren et al., 1997, 1999; Alexeev and Yoon, 1998; Alexeev et al., 2000; Bartlett et al., 2000). These investigations in mammalian cells revealed several findings about the chimera strategy: (a) the efficiency is high and exceeds that found in experiments with homologous recombination, (b) it provides a high specificity of nucleotide conversion or nucleotide incorporation, and (c) corresponding DNA/DNA duplexes remain inactive under the same conditions.

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In the plant kingdom, the production of chimera-mediated point and frame shift mutations in genomic (acetolactate synthase [ALS] gene) and in artificial (nonfunctional green fluorescent protein gene) targets, respectively, was described in somatic cells of tobacco (*Nicotiana tabacum*) and maize (*Zea mays*; Beetham et al., 1999; Zhu et al., 1999). However, in these studies, whole plants were regenerated only from maize callus. Segregation of herbicide-resistant and nonresistant phenotypes exhibited the expected Mendelian ratios (Zhu et al., 2000), which indicated that chimera-dependent changes could be stably transmitted through meiosis as well as mitosis. Unexpectedly, the application of chimera-mediated gene repair techniques in plants results in low efficiencies in comparison with animal cells. The appearance of semitargeted mutations as a shift of conversion from the expected nucleotide in the 5′ position or mutations other than those intended in the expected nucleotide also occur in plants. Studies in cell-free extract system by Rice et al. (2000) confirmed these peculiarities of chimera-dependent conversion in plants.

ALS catalyzes the initial step common to the biosynthesis of the branched chain amino acids Leu, Ile, and Val. Sulfonylureas are one class of herbicides that specifically inhibit this enzyme. In resistant lines, the herbicide insensitive form of ALS is present (Chaleff and Mauvais, 1984). The allotetraploid species tobacco has two genetically unlinked loci, *SuRA* and *SuRB*, for ALS. Mutations in either *SuRA* (Pro-196-Glu and Pro-196-Thr) or *SuRB* (Pro-196-Ser) locus result in single amino acid replacements at position 196 and a herbicide-resistant phenotype (Lee et al., 1988; Harms et al., 1992; Beetham et al., 1999). It was also shown that the S4-Hra mutant of tobacco bearing two linked mutations within locus *SuRB* had amino acid substitutions at positions 196 (Pro-196-Ala) and 573 (Trp-573-Leu), and it was more resistant to chlorsulfuron. Genetic linkage between these mutations did not allow the recovery of the independent contribution of each mutation for these highly resistant phenotypes (Creason and Chaleff, 1988). Lee et al. (1990) were able to target the wild-type ALS gene of tobacco via homologous recombination. To produce an amino acid substitution from Trp to Leu that would render a chlorsulfuron-resistant phenotype, a targeting vector carrying a 3′ end fragment of a mutant gene from S4-Hra was used. Although targeting was achieved, it was accompanied by random integration of the targeting vector into the genome, and the phenotype conferred by Hra mutation remained unknown. Interestingly, the same type of amino acid change (Trp to Leu) in the conserved region near the C terminus of ALS (as a result of 1-bp substitution, G to T) resulted in sulfonylurea resistance in a *Xanthium* sp. and *Brassica napus* (Bernasconi et al., 1995; Hattori et al., 1995). Moreover, in cocklebur (*Xanthium*), all possible mutations affecting the Trp at this position were investigated using site-directed mutagenesis, and only Trp-Leu substitution yielded an active, herbicide-insensitive form of ALS (Bernasconi et al., 1995).

In our study, we used an oligonucleotide-mediated strategy to create single point mutations at different positions within ALS genomic sequences of tobacco. Sequence analysis confirmed that application of oligonucleotides with various targeting sequences resulted in the production of predicted alterations. The same frequency of chimera-mediated conversions at different target sites suggests the absence of influence of genomic target sequence on the gene repair in tobacco. The assay of ALS activity in the leaves of resistant lines in the presence of chlorsulfuron demonstrated the appearance of an herbicide-insensitive form of the enzyme. Our data also suggest a correlation between the appearance of nonspecific chimera-dependent alterations and the ability of selection systems applied to detect them.

RESULTS

Production of Site-Specific Modifications to the ALS Gene by Chimera-Mediated Mutagenesis

In the allotetraploid species tobacco, there are two highly conserved ALS genes (*SuRA* and *SuRB*) that are expressed in all tissues (Keeler et al., 1993). Two chimeric RNA/DNA oligonucleotides were designed to obtain separate targeted single-nucleotide conversion at two different positions within either of these genes (Fig. 1, A and B). The chimeras used in our study had the same structure as those that directed repair mutations in animals (Kren et al., 1999; Alexeev et al., 2000; Bartlett et al., 2000). The targeting region of these chimeras consisted of a 25-bp region homologous to the target DNA with the exception of 1 centralized bp. ChALS-588 was designed to produce single nucleotide substitution from C to A at nucleotide position 588, and ChALS-1719 was designed to convert G to T at nucleotide position 1,719. These nucleotide conversions led to amino acid changes Pro-Gln at position 196 and Trp-Leu at 573, respectively. Both predicted amino acid substitutions, Pro-196-Glu and Trp-573-Leu, must result in a herbicide-insensitive form of ALS. Therefore, the desired conversion of the targeted base could be recovered by growing transformed cell colonies on chlorsulfuron-containing medium (Fig. 2A). Chimeric RNA/DNA oligonucleotides (ChALS-588 and ChALS-1719) were introduced into tobacco cells independently. In each experiment, several types of controls were used: untreated cells or protoplasts, treated without oligonucleotides, treated with nonspecific chimeric oligonucleotide, and treated with oligonucleotide composed of DNA only (ChALS-587). Electroporation of protoplasts and bombardment of cell colonies were used to

Figure 1. Sequences of targeting oligonucleotides and strategy of gene correction by chimeric oligonucleotides. A, Oligonucleotide sequences. B, Schematic presentation of chimera-mediated gene conversion. The endogenous DNA repair system mediates conversion of G to T at position 1,719, gives rise to substitution of Trp to Leu, and leads to appearance of chlorsulfuron-resistant phenotype. Lowercase letters indicate 2′-*O*-methyl RNA residues, and uppercase letters indicate DNA bases. The mismatched base is underlined. The chimeric ChALS-588 and the DNA-composed oligonucleotide ChALS-587 are designed to make conversions at the codon for Pro-196, whereas chimera ChALS-1719 is designed to alter the codon for Trp-573. ChALS(−) is a nonspecific chimera.

deliver the chimeric oligonucleotide ChALS-1719. Four colonies were recovered on selection medium with 140 nM chlorsulfuron after particle bombardment of 3×10^6 cells and five colonies after electroporation of 6×10^6 protoplasts. Then, we examined the capacity of the haploid state to promote chimera-mediated conversions. For this purpose, haploid lines of tobacco were generated by anther culture on double-layer medium H (Nitsch and Nitsch, 1969) with activated charcoal. The reduction in chromosome number was verified by caryological analysis (data not shown). The chimeric oligonucleotide ChALS-588 was delivered into protoplasts by electroporation. After treatment of 7×10^6 protoplasts of wild type and 6×10^6 protoplasts of haploid lines six and four, respectively, green-resistant colonies were found on medium containing 56 nM chlorsulfuron. In all control experiments (except cells treated with DNA oligonucleotide), 39×10^6 cells were used and two resistant colonies (Mu-1 and Mu-2) were obtained (but one colony was unable to grow when the selective agent in the medium was increased up to 140 nM chlorsulfuron). In experiments with a DNA-only oligonucleotide (ChALS-587), a total of 15×10^6 cells were treated, and two resistant colonies were recovered (rchl-17 and rchl-42).

Analysis of Targeted Sequences from the Resistant Lines

To confirm that resistant lines appeared due to chimera-dependent specific conversion, total DNA was isolated, and fragments including the target site near the 5′ or 3′ ends of the ALS gene were amplified by PCR. Direct sequencing of these fragments revealed a small new peak at the targeted codon on sequence chromatograms. The PCR products from wild type had unchanged sequence patterns (Fig. 3). The observed weak peak could be explained as artifacts or real conversion events that occurred only in one of several copies of ALS genes. To clarify this, we produced haploid plants from our resistant lines and selected them by growing on chlorsulfuron-containing medium. The idea was to decrease the number of unchanged copies of the ALS gene. Sequence analysis of the targeted region of resistant haploid lines revealed the presence of increased signal corresponding to the same nucleotide that was observed on chromatograms in the original T_0 plants. Furthermore, PCR products of targeted regions of T_0 resistant plants were ligated into the pPCR-Script Amp SK(+) vector and individually sequenced. In all cases, sequence analysis demonstrated the alteration of one nucleotide at the codon corresponding to Pro-196 or Trp-573. Because a change in nucleotides at

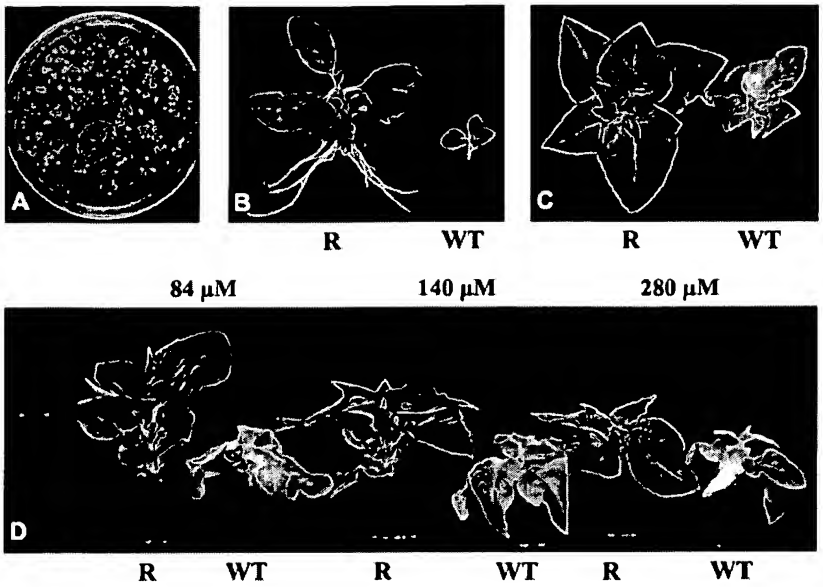

Figure 2. Resistance to chlorsulfuron at cellular and whole plant levels. A, Green-resistant colony on selective medium. B, Plants of wild-type (WT) and resistant (R) line rchl-6.6 growing on medium containing 560 nM herbicide. C and D, Response of resistant and wild-type plants to foliar application of chlorsulfuron.

different positions within the ALS gene could provide chlorsulfuron resistance, we performed sequence analysis of PCR fragments generated from the 5′ and 3′ ends of ALSs from all analyzed resistant lines to ensure that resistance was a result of chimera-mediated conversion. In the case of the chimeric oligonucleotide ChALS-1719, the observed change was limited to a single nucleotide substitution (G to T) at position 1,719; the codon for Pro-196 was unchanged. Application of the chimeric oligonucleotide ChALS-588 resulted in changes to the codon Pro-196 as predicted; no changes were found at the second possible region. Interestingly, in experiments targeting the Trp-573 codon, only predicted base replacements (G→T) were found. However, the second chimeric oligonucleotide (ChALS-588) targeting the Pro-196 codon also resulted in semitargeted conversions. Sequence analysis of two lines (rchl-17 and rchl-42) obtained using DNA-only construct (ChALS-587) resulted in single nucleotide conversions (C→G and C→A, respectively) at predicted positions only (Fig. 3; Table I).

Resistance on Cellular and Whole-Plant Levels

Callus cells remained chlorsulfuron resistant during several passages, demonstrating stable transmission of the modified gene in somatic cells. To induce organogenesis, resistant colonies were transferred to regeneration medium (Murashige and Skoog, 1962; 1 mg L^{-1} 6-benzyl-aminopurine, and 0.1 mg L^{-1} α-naphthaleneacetic acid) with 140 nM herbicide. Regenerated plantlets were cultured on Murashige and Skoog medium containing 420 nM chlorsulfuron. Wild type could not grow at such herbicide concentrations, but all resistant lines were able to produce roots and elongate shoots (Fig. 2B). The inhibition of root production was detected only for line rchl-3.11. T_0 plants were transferred into the greenhouse, and 3-week-old plants were sprayed with chlorsulfuron. Different concentrations (84, 140, and 280 μM) of herbicide were applied. Two weeks after treatment, wild-type plants were discolored, widely necrotic, and sometimes the whole leaf blade was dry (Fig. 2D). A concentration of 84 μM was sufficient to distinguish resistant and nonresistant phenotypes (Fig. 2C). Resistant lines were significantly different from the wild type in fresh weight. Plants of resistant lines had green leaves, and no necroses were detected except for plants of line rchl-3.11. Some leaves of these plants had small necrotic spots (Table II).

It is worth noting that during regeneration of resistant colonies that were selected in the experiments with haploid plants, chromosome doubling took place (data not shown). Hence, regenerated dihaploid plants were homozygous for the altered ALS gene that led to higher viability after herbicide treatment (Table II).

Biochemical Analysis

To prove that chimera-mediated conversions resulted in the appearance of a chlorsulfuron-insensitive form of ALS, enzyme activity was assayed in the presence and absence of chlorsulfuron. ALS activity in the leaf extracts from wild type was greatly inhibited under all tested concentrations. All resistant lines demonstrated lower sensitivity of ALS in extracts to the inhibition by chlorsulfuron. Line rchl-3.11 was intermediate in its response to increasing herbicide concentrations in comparison with other lines that demonstrated similarly high degrees of resistance to chlorsulfuron (Fig. 4).

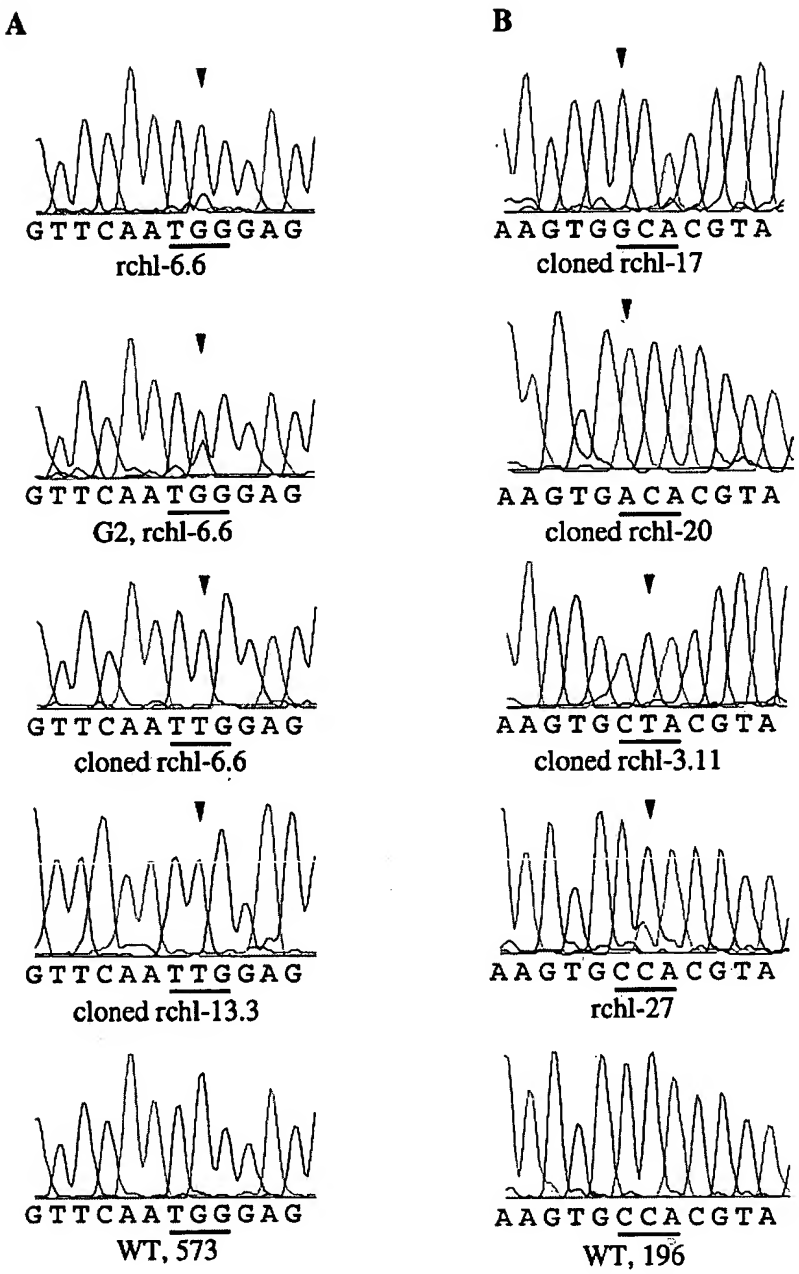

Figure 3. Confirmation of oligonucleotide-mediated acetolactate syntase gene conversion by sequence analysis. Nucleotide sequences from control wild type (WT) and independent resistant lines rchl-6.6, rchl-17, rchl-20, and rchl-3.11 are shown. G2 rchl-6.6 is sequence of haploid from line rchl-6.6. Plate A, Nucleotide substitutions at the codon for Trp-573. Plate B, Nucleotide substitutions at the codon for Pro-196. The targeted codon is underlined and conversion events are indicated with arrows.

Transmission of the Altered Gene through Meiosis

Seeds from self-fertilized resistant lines (T_0 plants) were collected and after germination were transferred to the medium containing 560 nM chlorsulfuron. T_1 seedlings segregated for resistant and nonresistant phenotypes (Table II). On average, about one-third of the seedlings were sensitive to the herbicide. These results indicated that chlorsulfuron resistance was inherited as a single dominant Mendelian trait.

DISCUSSION

Homologous recombination-dependent gene targeting in plants is characterized by low frequency of integration and random integration of targeting vector elsewhere in the genome (Morton and Hooykaas, 1995; Mengiste and Paszkowski, 1999). In addition, some examples of mitotic and meiotic instability of targeted genes have been described (Paszkowski et al., 1988; Lee et al., 1990; Schaefer and Zryd, 1997). In contrast, oligonucleotide-directed gene targeting is not accompanied by random construct integration, and converted genes are regulated by their own promoters in their natural genetic background (Kren et al., 1997; Zhu et al., 2000). Altered genes are also stably maintained during mitosis (Alexeev and Yoon, 1998; Beetham et al., 1999; Igoucheva and Yoon, 2000) and transmitted in

Table I. *Summary of chimera-directed alterations of ALS gene*

Target (Position of Nucleotide)	Line	Predicted Changes		Observed Changes	
		Nucleotide	Amino acid	Nucleotide	Amino acid
ALS-588	rchl-27	CCA→CAA	Pro-196-Gln	CCA→CAA	Pro-196-Gln
	rchl-3.11			CCA→CTA	Pro-196-Leu
	rchl-20.1			CCA→ACA	Pro-196-Thr
	rchl-5.5			CCA→TCA	Pro-196-Ser
ALS-1719	rchl-6.6[a]	TGG→TTG	Trp-573-Leu	TGG→TTG	Trp-573-Leu
ALS-587	rchl-17				
	rchl-4.2	CCA→GCA	Pro-196-Ala	CCA→GCA	Pro-196-Ala
				CCA→ACA	Pro-196-Thr

[a] All lines have the same substitution.

a Mendelian fashion to subsequent generations (Zhu et al., 2000).

In this study, we report the use of synthetic RNA/DNA oligonucleotides to produce distinct, precise modifications within the ALS gene of tobacco at two different target sites. Two chimeras were designed and delivered in independent experiments into wild-type cells of tobacco. Such an approach should allow us to distinguish between chimera-mediated conversions and random mutations. In each set of experiments in which different chimeric oligonucleotides were used, detection of conversion event at the targeted region and absence of any changes at the second possible site served as additional proof that the herbicide-resistant phenotype appeared as a result of chimera-directed gene targeting. On the other hand, this should answer the question about the possibility of a single mutation within the ALS gene at nucleotide 1,719 by itself to provide chlorsulfuron resistance, and if this is the case, about the degree of this resistance. A previously described double mutant of tobacco S4-Hra bearing two linked mutations in Pro-196-Ala and Trp-573-Leu possessed a highly chlorsulfuron-resistant phenotype (Creason and Chaleff, 1988; Lee et al., 1988). Because one mutation was produced in the genetic background of another, it was not possible to examine the contribution of each of them in the resulting phenotype. Our experiments with chimera ChALS-1719 clearly demonstrated that single amino acid substitution Trp-573-Leu alone can provide a chlorsulfuron-resistant phenotype. The fact that this mutation was generated on the wild-type background allowed its phenotypic expression to be revealed. As follows from results of enzyme activity assays, two resistant lines, rchl-17 (Ala-196 corresponding to S4 mutation) and rchl-6.6 (Leu-573 corresponding to Hra mutation), possessed similar level of ALS activity (Fig. 4) and responded in similar manners to herbicide treatment (Table II). These data suggest that the high level of chlorsulfuron resistance seen for the double mutant S4-Hra probably arose from an additive effect of two mutations.

Application of only DNA-composed oligonucleotide ALS-587 resulted in the generation of alterations at the intended nucleotide (Table I; Fig. 3). However the frequency of changes was low. There are two

Table II. *Assessment of wild-type and independent resistant lines after herbicide application and segregation of chlorsulfuron resistance in T_1 progeny*

Line	Response on Application of 84 μM Chlorsulfuron				T_1 Progeny Seedlings No. of Plants	
	Plant phenotype	Leaf color	Presence of necroses	Fresh weight[a]	Resistant	Sensitive
				g		
WT (Pro-196 and Trp-573)	Sensitive	Bleached	+++	197.02 ± 8.36 a	0	330
rchl-3.11 (Leu-196)	Susceptible	Green	+	214.6 ± 12.87a	149	61
					χ^2 = 1.835	
rchl-20[b] (Thr-196)	Resistant	Green	–	316.25 ± 9.27 c	400	0
rchl-17 (Ala-196)	Resistant	Green	–	262.02 ± 5.31 b	113	37
					χ^2 = 0.009	
rchl-5.5 (Ser-196)	Resistant	Green	–	254.12 ± 6.23 b	167	53
					χ^2 = 0.097	
rchl-6.6 (Leu-573)	Resistant	Green	–	242.17 ± 15.92b	184	68
					χ^2 = 0.529	
rchl-2.5 (Leu-573)	Resistant	Green	–	237.02 ± 14.01b	175	65
					χ^2 = 0.556	

[a] Values presented as a mean ± SE from measurements of five independent plants. Within-column means without common letters are significantly different at $P < 0.05$. [b] Resistant dihaploid line. +, Indicates that some leaves had small necrotic spots; +++, indicates wide necroses up to dryness of the whole leaf blade. χ^2 values were calculated on the basis of expected ratio of three resistant to one sensitive plant.

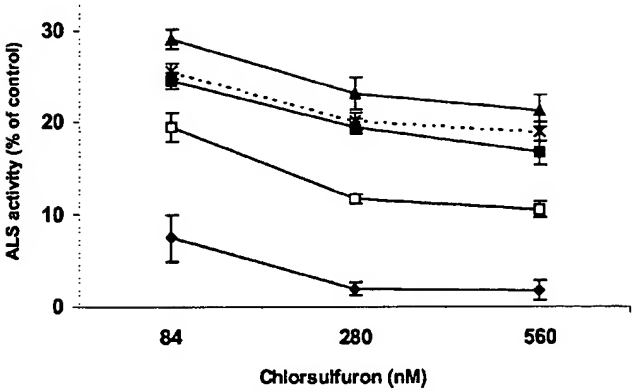

Figure 4. Inhibition of ALS activity by chlorsulfuron in leaf extracts. ALS activities are presented as percentages of the respective activity in the absence of herbicide. ▲, Line rchl-6.6; *, rchl-5.5; □, rchl-3.11; ■, rchl-17; ◆, wild type. Each data point is the mean ± SE from measurements of activity of five independent plants.

lines of evidence that a DNA duplex was able to provide repair activity in vivo. First, changes were found in the codon for Pro-196, which was chosen as a target, but a second possible place (codon Trp-573) alteration, which also could result in generation of herbicide resistance, remained unchanged. Second, even alterations in the codon Pro-196 at any nucleotide could lead to production of herbicide-resistant phenotype. However, only the targeted nucleotide was modified, which also indicated oligonucleotide-directed character of conversions. This is consistent with findings in the in vitro system (plant cell-free extracts), where it was shown that all-DNA oligonucleotides mediated targeted repair (Rice et al., 2000). In contrast, such activity has never been observed in experiments with mammalian cells (Yoon et al., 1996; Kren et al., 1997).

Using both chimeric oilgonucleotides resulted in the intended conversions of the ALS gene of tobacco; however, some semitargeted alterations also were detected (Table I). These results are contradictory to those obtained in a study by Beetham et al. (1999), where a chimera-mediated change of the codon for Pro-196 in the ALS gene of tobacco was achieved, but in all cases the only nucleotide located near the 5′ end of the targeted base was changed. In our opinion, detection of only semitargeted modifications in the same model system could reflect peculiarities of the selection system used (see below). The big pool of spontaneous mutations, which was observed in this work, and the inability to regenerate plants could be referred to using tobacco cell suspension culture. Reduction of morphogenic potential and appearance of mutations during maintenance of cell suspension cultures is well documented (Halperin, 1986; Sree Ramulu, 1986). Our results showed that mesophyll protoplast cells are competent in the chimera-mediated conversions. Using mesophyll protoplasts also allowed the reduction of the appearance of spontaneous mutations and the regeneration of plants to be achieved. This made possible further study of the inheritance of the modified gene.

In experiments with maize, a precise targeted correction mediated by a chimera was reported, although additional semitargeted conversions were also found (Zhu et al., 1999). Using a plant cell-free extract system, the diminished fidelity of chimera-directed gene repair (incorrect conversion at the predicted nucleotide position as well as a shift in the 5′ side from the targeted nucleotide) was confirmed (Rice et al., 2000). In vitro and in vivo experiments with plant semitargeted conversions were always detected at one position in 5′ direction to the targeted nucleotide. The shift of conversion in 3′ side was never observed. Because formation of a complement-stabilized D-loop is a key step in chimera-mediated gene repair, the appearance of these non-correct mutations could be explained by the existence of alternative pathways (high-fidelity repair and different mutagenic pathways) for processing this joint molecule (Gamper et al., 2000a). The correlation between appearance of unspecific base alterations and nonoptimal modifications of the backbone of the chimera (Gamper et al., 2000a) or single-stranded oligonucleotides (Gamper et al., 2000c) also supports this assumption. Interestingly, the frequency of conversion events for chimera-mediated repair in plants was 3 orders of magnitude less than was observed for mammalian cells (Zhu et al., 1999). In combination with observations that a DNA duplex can provide targeted gene conversions and that chimera-dependent nonspecific mutations can be generated, this finding indicates the possible existence of different repair pathways in animals and plants.

In our experiments, a second nucleotide at the codon corresponding to Pro-196 or Trp-573 was chosen as a target for chimera-mediated conversion. Such an approach should allow us to detect both types of previously revealed nonspecific alterations as a shift of conversion and incorrect conversion if those would take place and distinguish oligonucleotide-dependent alterations and random mutations. Surprisingly, chimera ChALS-1719 led to only targeted conversion, whereas application of ChALS-588 resulted in both targeted and semitargeted alterations (Table I; Fig. 3). In our opinion, this can be explained by the selection system applied. Recovery of conversion events in a big pool of treated cells was based on expression of the altered form of an ALS gene, the product of which renders tobacco cells resistant to the herbicide. Several single amino acids substitutions at position 196 of ALS, Pro-196-Glu, Pro-196-Ser, Pro-196-Ala, and Pro-196-Thr (Lee et al., 1988; Harms et al., 1992; Beetham et al., 1999) and Pro-196-Leu (this study, Table I) led to the appearance of chlorsulfuron-resistant phenotypes. Thus, any chimera-dependent nucleotide changes at this codon (no matter if the first or second nucleotide was changed) could be recovered. In contrast, within

other conserved sites of the ALS protein involved in the herbicide binding, only one specific amino acid substitution, Trp-573-Leu, led to sulfonylurea resistance (Creason and Chaleff, 1988; Bernasconi et al., 1995; Hattori at al., 1995). Therefore, even though other types of alteration took place in the experiments with ChALS-1719, these would not have been recovered because they were not accompanied by chlorsulfuron resistance. The results obtained with all-DNA construct (ChALS-587) also support such conclusions. A shift of repair conversion in the 5′ side from the predicted nucleotide was described (Beetham et al., 1999; Zhu et al., 1999; Rice et al., 2000). Thus, theoretically we could expect such an effect, but only conversions at the targeted nucleotide 587 were obtained (Table I). The substitution of C for G or A can be understood because any nucleotide substitutions for the first nucleotide of the Pro-196 codon could be identified using our selection system (which is based on recovery of chlorsulfuron-resistant cells). In contrast, even if the third nucleotide (which is at a 5′ side of the targeted nucleotide) of the adjacent codon of Val-195 is changed to another nucleotide, it would result in a silent mutation, and such an event would remain undetectable because it would not render resistance to the herbicide.

In previous studies, the appearance of chimera-directed conversions were proved on the DNA level by RFLP and sequence analyses. Together with the latter, we also used enzyme activity assay to verify conversions at the protein level. Variable levels of ALS resistance to chlorsulfuron among different mutant lines were revealed (Table II; Fig. 4). Thus, the line rchl-3.11 containing substitution Pro-196-Leu had approximately 2-fold less resistance to chlorsulfuron than other lines. This was accompanied by stronger inhibition of callus, seedling growth, and delay of root production on selective medium. The observed differences suggest that different types of amino acid substitution confer unequal levels of chlorsulfuron resistance, and, under strong selective conditions, cells with defined type of alterations could not survive. This finding explains why in the study of Beetham et al. (1999), after application of a chimeraplast designed to modify codon for Pro-196 (CCA to CTA), only semitargeted alterations were detected. In that report, regeneration of herbicide-resistant plants was not achieved and conversion events were identified as calli that were able to grow under strong chlorsulfuron selection. Our data clearly show that under such conditions, cells bearing Leu (CTA) at the position 196 in the amino acid sequence of ALS are susceptible (Table II; Fig. 4). Thus, the selection system applied in those experiments could be one of the reasons why only conversions adjacent to the targeted nucleotide were observed. Zhu et al. (1999) demonstrated that chemical selection influenced the frequency of chimera-mediated alterations. In maize, the frequency of green fluorescent protein-expressing cells that appeared because of conversion events was higher in comparison with experiments where chemical selection was employed. These data and our results demonstrate that the selection system applied may influence the frequency of chimera-mediated targeting events and the ability to detect semitargeted chimera-dependent conversions.

Studies with yeast, bacteria, mammalian, and plant cells did not reveal obvious restriction to DNA sequences that can be targeted by chimeric oligonucleotides (Graham and Dickson, 2002; Kren and Steer, 2002, and refs. therein). However, when the APOAI gene in human (*Homo sapiens*) HepG2 cells was targeted at two locations, the conversions were obtained only at one target site ($apoAI_{Paris}$). A limited correction at second site ($apoAI_{Milano}$) was achieved in CHO cells. This finding pointed out the possible limitation of chimera-mediated correction by differences between the two target sequences (Graham et al., 2001). In our experiments, application of ALS-588 and ALS-1769 chimeraplasts, mediating conversion at two different sites inside the ALS gene, resulted in comparable frequency of conversion events. Together with the fact that our chimeras had the equal purity and the same level of GC content, this suggests that the influence of genomic target sequence on the gene repair in tobacco was not detected.

Gene targeting of plants using homologous recombination is currently limited by low efficiency and inaccurate integration. Only moss (*Physcomitrella patens*) demonstrates a high rate of homologous recombination. It has been speculated that the efficiency of homologous integration might correlate with the haploid state (Schaefer and Zryd, 1997). The mechanisms of chimera-directed gene repair and homologous recombination are different (Gamper et al., 2000a; Chen et al., 2001). However, experiments with chicken (*Gallus gallus*) B cells and embryonic mouse (*Mus musculus*) fibroblasts lacking an active p53 protein in which high frequency of homologous recombination was correlated with high frequency of chimera-mediated conversion demonstrated that cell recombination activity is important for chimera-dependent repair (Igoucheva et al., 1999; Alexeev and Yoon, 2000). The observation that the RecA protein can catalyze strand exchange between chimera and DNA duplex also suggests that recombination is a necessary step in chimera-dependent correction (Gamper et al., 2000a, 2000b). In view of these results, we assessed the role of the haploid state in increasing frequency of chimera-mediated conversion. For this purpose, haploid plants of tobacco were produced, and chimera ChALS-588 was electroporated into protoplasts. However, the number of recovered chlorsulfuron-resistant colonies did not differ significantly from those derived from wild-type protoplasts (see results). This observation is consistent with studies on the haploid green alga *Volvox carteri*

and haploids of tobacco in which it has been shown that a haploid nature per se was not enough for successful targeting of arylsulfatase gene (Hallmann et al., 1997) or disruption of the nitrate reductase gene (Lebel, 1994). Although our results suggest that the haploid state by itself could not elevate the frequency of targeted conversion, using haploids in chimera-targeting experiments provides some new insights. First, it gives opportunity to operate with recessive traits, and second, it makes possible one-step production of homozygous plants with respect to the modified gene.

CONCLUSION

In summary, we produced chlorsulfuron-resistant lines of tobacco by modifying an endogenous ALS gene at different positions using in vivo site-specific oligonucleotide-mediated conversion. The limitation of chimera-mediated gene conversion by differences in genomic target sequences was not observed. Using this technique allowed the generation of plants with separate new point mutations causing amino acid substitutions Pro-196-Leu and Trp-573-Leu, which are characterized by different phenotypic and biochemical responses to the application of chlorsulfuron. The selection system applied may influence the capacity to detect different types of chimera-mediated alterations. Stable transmission of chlorsulfuron resistance in several generations of somatic cells of tobacco and Mendelian inheritance in T_1 progeny verified the permanent character of chimeroplast and all-DNA oligonucleotide-dependent conversions.

The low frequency of observed alterations will still discourage wide application of this strategy. However, we hope that further elucidation of mechanism of oligonucleotide-mediated targeted gene conversion would help to improve the design of structure of RNA/DNA or single-stranded oligonucleotides that would result in increase of frequency. This would allow the expansion of the applicability of this technique as a powerful tool for the modification or disruption of the genes to create desirable phenotypes or knockout mutants.

MATERIALS AND METHODS

Design of Targeting Oligonucleotides

The oligonucleotide sequences (Interactiva, Ulm, Germany) are shown in Figure 1. They were designed to target the ALS gene of tobacco (*Nicotiana tabacum*) at the desired position and, with the exception of ChALS(−), shared homology to this gene. Chimera ChALS-1719 was designed to create a single G→T substitution at nucleotide position 1,719, and ChALS-588 was designed to create an A→C conversion at position 588. Oligonucleotide ChALS-587 was constructed to produce a single C→G conversion at position 587 and was composed of DNA residues only. The ChALS(−) chimeric oligonucleotide had no homology to the ALS sequence and served as a nonspecific control.

Plant Material and Transformation Procedure

Axenic shoots of tobacco cv Samsun were cultivated on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 20 g L^{-1} Suc at 25°C, with illumination of 250 μmol $m^{-2}s^{-1}$ and a 16-h photoperiod. Mesophyll protoplasts were isolated using enzyme medium containing 1% (w/v) Cellulase R10 (Sigma, St. Louis) and 0.5% (w/v) Driselase (Fluka) in a solution of 0.5 M Suc and 5 mM $CaCl_2$. After overnight incubation of the aseptic leaves in this mixture at 25°C, the suspension was diluted by 0.5 M Suc, passed through nylon mesh (100 μm), and centrifuged at 50*g* for 5 min. Floating protoplasts were collected and washed twice with W5 solution (Menzel and Wolfe, 1984). Isolated protoplasts (5×10^5) were electroporated with the appropriate oligonucleotide (0.5 μg) using a Bio-Rad Gene Pulser II (Bio-Rad Laboratories, Hercules, CA) in medium M (0.3 mL) containing 8 mM HEPES, 4 mM $CaCl_2$, 70 mM KCl, and 0.4 M mannitol (pH 7.2). Electroporation conditions were as follows: voltage, 250 V; capacitor, 240 μF; and distance between electrodes, 4 mm. Protoplasts were cultivated in dark conditions at 25°C in 10 mL of K_3NM medium (Nagy and Maliga, 1976) containing 0.4 M mannitol as an osmotic stabilizer. After 7 d, they were transferred to light conditions. Selection was started in liquid culture 2 weeks after protoplast isolation. Then, cell colonies were transferred to solidified selective medium with 56 and 140 nM chlorsulfuron for ChALS-588 and ChALS-1719, respectively. In particle bombardment experiments, protoplasts were cultured as described above and colonies about 1 to 2 mm in diameter were used for bombardment. Oligonucleotides (0.5 μg) were precipitated onto gold particles (1 μm) and delivered to microcalli using a Bio-Rad PDS-1000 He device as described previously (Klein et al., 1988). A helium pressure of 1,100 psi was used to accelerate the particles, and the vacuum in the chamber was 28 inches of Hg. After bombardment, colonies were plated on Murashige and Skoog medium with 0.2 M mannitol and were transferred in 2 or 3 d onto selective medium with 140 nM chlorsulfuron and without mannitol. After 3 to 4 weeks of culture, resistant colonies were transferred to Murashige and Skoog regeneration medium containing 1 mg L^{-1} 6-benzyl-aminopurine and 0.1 mg L^{-1} α-naphthaleneacetic acid.

Haploid Plant Production and Caryotype Analysis

Anthers of tobacco were cultured on H-medium (Nitsch and Nitsch, 1969) using a double-layer method (Johansson et al., 1982). Root tip cells from the aseptically grown, haploid plants produced using this method were caryotyped as described previously (Kochevenko et al., 1996).

PCR Amplification and Sequencing Analysis

PCR was performed with Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) to ensure high fidelity. Reactions were carried out in 50-μL volumes containing 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% (v/v) Triton X-100, 200 μM of each dNTP, 250 ng of each primer, and 100 ng of genomic DNA. After an initial denaturation step at 95°C for 5 min, the reaction mixtures were subjected to 35 amplification cycles of 95°C for 1 min, 65°C for 1.3 min, and 72°C for 2 min with subsequent incubations for 10 min at 72°C. The sequences of the primers used were (5′ to 3′): 1fwd, GGGTTACGCACGCGCCACCGG; 1rev, GGCTGATCCCAGTCAGGTATC; 2fwd, CACCAGATGTGGGCTGCTCAA; and 2rev, GCAGCAGGTACGCCACAAGCC. Amplification products were separated by electrophoresis on 1% (w/v) agarose gels, stained with ethidium bromide, and visualized under UV light. The amplified fragments with expected size were recovered and sequenced directly or subcloned into the pPCR-Script Amp SK(+) vector (Stratagene) for subsequent DNA sequence analysis. DNA sequencing was done by AGOWA GmbH (Berlin).

Assay of ALS Activity

Plants were grown in a greenhouse at 26°C, with illumination of 250 μmol $m^{-2}s^{-1}$, and under a 16-h photoperiod. Leaf samples were harvested from 4-week-old plants and immediately frozen. One gram of leaf material was homogenized in 8 mL of extraction buffer containing 100 mM potassium phosphate buffer (pH 7.5), 0.5 mM $MgCl_2$, 1 mM sodium pyruvate, 0.5 mM thiamine pyrophosphate, 10 μM FAD, 10% (v/v) glycerol, and 1% (w/v) polyvinylpolypyrrolidone (PVPP). Extracts were prepared and ALS activity

was assayed as described by Chaleff and Mauvais (1984). Inhibition of ALS activity was measured in the presence of 84, 280, and 560 nM chlorsulfuron. The protein content in the same extracts was measured by the method of Bradford (1976).

Growth Test for Herbicide Resistance

Herbicide-resistant phenotypes were studied under greenhouse conditions after foliar application of chlorsulfuron. Three-week-old plants were sprayed with a solution (10 mL per plant) containing 0.2% (v/v) Tween 20, 10% (v/v) acetone, and the herbicide (84, 140, or 280 μM of chlorsulfuron). Plant fresh weight was determined 2 weeks later.

Segregation Analysis

Regenerants were self-fertilized in the greenhouse. Obtained seeds were surface sterilized and after germination, seedlings were transferred to Murashige and Skoog medium containing 560 nM chlorsulfuron. Plants showing root and shoot growth were scored as resistant.

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